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X-RAY EFFECTS ON HEMOLYSIN FORMATION IN RABBITS WITH THE SPLEEN SHIELDED OR IRRADIATED

WILLIAM H. TALIAFERRO AND LUCY GRAVES TALIAFERRO

From the Department of Microbiology, The University of Chicago, Chicago 37, Illinois

Jacobson et al (1950) were the first authors to study the protective effect on hemolysin production of lead-shielding the surgically exteriorized spleen of the rabbit receiving severe X radiation to the rest of the body. Later, Jacobson and Robson (1952) reported that the capacity to form hemolysins in rabbits receiving 800 r or 500 r X radiation with the spleen similarly shielded was retained when the shielded spleen was removed 24 hours after irradiation and the sheep red cell antigen was injected 48 hours after irradiation and 24 hours after splenectomy. They suggested that the protective effect was the result of the functional restoration of cells in the body following the liberation of some humoral substance into the circulation by the spleen during the 24 hours between irradiation and splenectomy. In both papers by Jacobson and his associates, a single intravenous injection of sheep red cells was given to each animal. Wissler et al (1953) found a marked protective effect on hemolysin production in the rat by lead-shielding the spleen while the rest of the body received 500 r. This protection was obtained whether the sheep red cells were given intraperitoneally or intravenously. These papers will be considered in more detail in the Discussion.

The present investigation was carried out with the following objectives in mind: (1) to repeat the work of Jacobson and his associates, using the more sensitive and quantitative technique of titrating hemolysin in 50% hemolytic units and our methods of obtaining antibody curves for each animal, which permit various determinations of the character of the immune response, and (2) to compare the effects of lead-shielding the spleen while irradiating the rest of the body with the reverse of lead-shielding the body while irradiating the spleen. As regards the first objective, our conclusions differ in certain respects from those of the previous authors. As regards the second objective, the most important result is that large doses of X rays, administered locally to the exteriorized spleen, stimulate the production of above normal amounts of hemolysin at slightly increased rates within normal periods of time.

In the present study, the following characteristics of rabbit antisheep hemolytic antibodies should be kept in mind. A single intravenous injection of whole sheep red cells, as used in this study, results in the formation of Forssman antibody. (Isophile antibody only develops later after repeated injections.) The Forssman antibody can be absorbed on sheep red cells and is

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composed of two fractions of related, if not identical, specificity. The first is highly hemolytic, has a molecular weight of about 900,000 and a biological half life of 2.8 days. The second is only slightly, if at all, hemolytic, has a much smaller molecular weight and a biological half life of 5.6 days (Taliaferro and Talmage, 1956). Our titration technique measures almost exclusively the large molecular hemolytic component.

MATERIALS AND METHODS

Rabbits.—The rabbits used at first were infected with *Eimeria stiedae* and *Eimeria perforans* and frequently suffered from respiratory infections. These are designated as Sharp rabbits. Most of the rabbits, however, were free of respiratory infections as well as *Eimeria*. They were obtained from Mr. Harold Swift's farm at Lakeside, Michigan. Males and females were used in about equal proportion and generally weighed between 2 and 2.5 kg at the beginning of an experiment.

Radiation.—The X-ray constants are given in table 1. They varied not only because we used two machines but because the surgically prepared animals could not be immobilized successfully for a period much longer than half an hour (cf last column in table 1). In irradiating the exteriorized spleen with 10,000 r, it was necessary to obtain part of the high dosage by halving the copper filter used for most of the lower doses. To integrate the results from 10,000 r with those of most of the smaller doses, a second experimental series with 5000 r was irradiated with the same filters employed for 10,000 r. There was no significant difference noted between the two series (A10 and A11). Of the 3 groups irradiated with different factors, and given 1400 r, Bartlett's test for homogeneity of variance indicated that the differences between the mean log peak titers could be a sampling variation ($P=0.3$), but also indicated that the rates of antibody rise varied significantly ($P<0.001$).

Lead shields.—The lead spleen shield used at first was kindly lent us by Dr. Jacobson and is described in detail by Jacobson et al (1950). Later, a shield* was used which served for the appendix as well as the spleen (fig. 1). It was made square in cross section so that it did not move easily and the edges of the baffle through which the blood vessels passed were well rounded. Both of these features protected the blood vessels. The open end necessary for use in appendix-shielding was supplied with a lead stopper for spleen-shielding.

The lead shield* to protect the body while irradiating the surgically exposed spleen consisted of 2 curved lead half shields which when fastened together with 2 springs contained a small central depression designed to hold the spleen (fig. 2). This shield also contained a protective baffle. The combined half shields, 0.6 cm thick, measured 7.5 cm wide and curved over a diameter of 10.5 cm to fit the body of the rabbit. The depression was 2 cm in diameter and 1.5 cm deep.

Surgical techniques in connection with irradiation.—Throughout this paper, series A designates

* We are indebted to Mr. Charles E. Soderquist of the Research Institutes of the University of Chicago for designing these shields and to Mr. John Hanacek of the same Institutes for making them.

TABLE 1.—*X-ray dosimetry.*

Series including controls except B5 and C5	Location of machine at the University of Chicago*	X-ray dose	Ma	Filter (mm)		Half layer value of filtered beam (mm copper)	Target distance from center of rabbit in cm	r per minute	Minutes of exposure
				Cu	Al.				
1, 16 (part)	USAF	700 r	15	0.25	1	0.9	50	18–21	33–39
2, 5, 16 (part)	Argonne	700 r	22	0.5	1	1.43	84.5	29–36	20–24
3	Argonne	700 r	30	0.5	1	1.43	125.5	18.5	38
4	Argonne	500 r	22	0.5	1	1.43	125.5	16–17	29–31
6, 7, 8	Argonne	500 r	30	0.5	1	1.43	125.5	19–21	24–26
9	Argonne	10,000 r	30	0.25	1	1.04	37.2	241–257	39–43
10	Argonne	5,000 r	30	0.25	1	1.04	50.8	119–125	39–43
11	Argonne	5,000 r	30	0.5	1	1.43	38.2	185–199	24–27
12	Argonne	2,600 r	30	0.5	1	1.43	56.2	104–110	24–27
13	Argonne	2,000 r	30	0.5	1	1.43	65.0	76–82	24–27
14	USAF	1,400 r	15	0.25	1	0.9	25.0	59–69	20–24
	Argonne	1,400 r	22	0.5	1	1.43	84.5	30–32	43–47
	Argonne	1,400 r	22	0.5	1	1.43	75.3	40–42	33–35
15	Argonne	1,000 r	30	0.5	1	1.43	85.0	37–43	24–27
17	Argonne	500 r	30	0.5	1	1.43	79.0	53–55	9–9.5

* The United States Air Force machine delivered the beam horizontally and the Argonne Cancer machine delivered it vertically; both operated at 250 kilovolts.

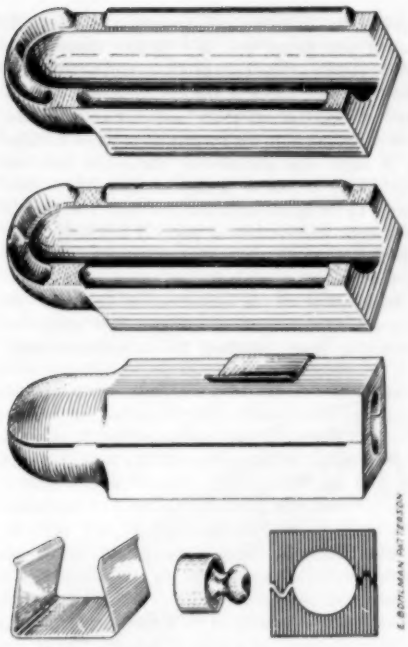

FIG. 1.—Various aspects of the combination spleen-appendix lead shield. ×½.

rabbits whose spleen alone was either X-rayed or shielded from X rays, whereas the control B and C series refer to rabbits shielded from Xrays or X-rayed in toto, respectively. Other control series, D and E, involve splenectomy with or without irradiation.

Except for one series of rough spleen-shielded rabbits (A5) and its accompanying controls (B5 and C5), all rabbits were spleen-shielded with operative techniques 2 days before the injection of sheep red cells. The rough spleen shield technique consisted of placing a 6×23 cm curved piece of lead, 0.6 cm thick, over the body of the rabbit in the region of the spleen. The controls were simply injected with red cells with (C5) or without (B5) total body irradiation.

The operative spleen-shielding technique involved starving the rabbit for 24 hours beforehand, opening the abdominal cavity under nembutal and ether anesthesia on a portable animal board, tieing off the small vein draining the distal end of the spleen, clamping the 2-part shield (fig. 1) over the spleen wrapped in wet gauze, covering the operative site with wet gauze and then turning the animal over so that the shield would rest on the board during X-ray treatment. The blood vessel at the distal end of the spleen was tied off to straighten the spleen out and allow it to fit in the lead shield better. This portion of the spleen was congested to varying degrees at

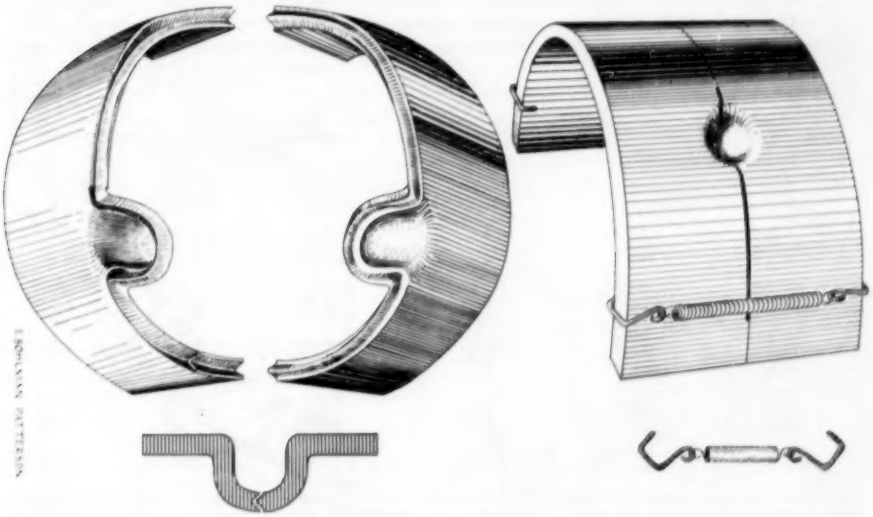

FIG. 2.—Various aspects of the lead shield which was used to shield the body and expose the spleen to X rays. After this shield was applied, 2 semicylindrical lead shields were added to cover the rest of the body of the rabbit. ×½.

the end of the irradiation period. Before closing the incision, 3 or 4 ml of antibiotic saline were poured into the body cavity. Ether was only used just before opening the body cavity. Otherwise, nembutal in an intravenous dose of about 30 mg per kg ordinarily kept the animal immobilized during the operation and the X-ray procedure. Antibiotic saline solution (0.85% NaCl) was used throughout the operative techniques and contained 1600 units of penicillin and 4 mg of streptomycin per ml.

Two types of controls accompanied each series of spleen-shielded rabbits. The nonirradiated type was treated as follows 2 days before the injection of red cells. After the body cavity was opened, the distal vein of the spleen was tied off, and a wet gauze was tied tightly around the blood vessels at the base of the spleen to simulate the traumatic effect of the spleen shield. Then a lead shield, 0.6 cm thick and cut transversely and at an angle to the surface to prevent leakage of X rays, was placed over the entire body to shield from a dose of X rays comparable to that given to its spleen-shielded partner. The irradiated type involved total body X irradiation, at which time the body cavity was opened, etc., as in the preceding control.

Irradiation of the spleen alone in series A9 through 17 was carried out with the operative technique just described in conjunction with

FIG. 3.—The regular scale (solid lines and figures) and the [log $y/(1-y)$]+1 scale (dotted lines and figures) on the Klett-Summerson colorimeter dial. The regular scale was used for standardizing all red cell suspensions, whereas the added scale was only used for titrating serums (see text and table 3).

lead half shields (fig. 2) to hold the spleen swathed in several thicknesses of wet gauze. The rest of the rabbit was then covered with the 2-part lead shield described above. The aperture emitting the X rays was partially shuttered to localize the X rays falling on the exposed spleen. The control series (B9–17) involved shielding the whole body of the rabbit from a dose of X rays comparable to that given to the spleen-X-rayed rabbits during which the body cavity was opened, etc., as previously described. Three to 6 spleen-irradiated rabbits were accompanied by 2 nonirradiated controls. For irradiated controls, those in section C of experiment 1 were considered adequate.

Immunization.—The suspensions of washed sheep red cells for injection were photometrically standardized on the basis of hemoglobin liberated by distilled water. One ml of such a suspension plus 4 ml of distilled water read 150 on the regular scale (fig. 3) of the Klett-Summerson colorimeter (=KS) with a 54 filter (500 to 700 mμ) and is equivalent to 1 ml of a 1% suspension or 1.6×10^8 red cells. The amount injected, i.e., 1.25 ml of the standardized suspension per kilogram rabbit, is designated for convenience as 2×10^8 red cells or 1.25 ml of a 1% suspension. The cells were kept in the refrigerator in Alsever's fluid until just before standardization and injection. They were washed in an excess of 0.85% NaCl solution.

Serological titrations.—All serums were titrated in 50% hemolytic units, defined as the amount of serum which hemolyzes 50% of a photometrically standardized suspension of red cells in the presence of an excess of complement (≈four 50% units of complement). The standardized suspension of red cells consisted of 2 ml of the suspension standardized as described in the preceding section but the cells were washed and suspended in veronal buffered saline (=VB) containing calcium and magnesium ions (Mayer et al, 1946 and 1947). The titration methods have been described in detail by us (1950), but a few changes should be mentioned.

Serums collected during one week were routinely tested the following week and were retested, if necessary, a week later. A 1:100 dilution of each antiserum (0.1 ml serum+9.9 ml VB in test tubes) was made and can be kept in the refrigerator a week or more without any discernible loss in titer. A new 1:100 dilution was made, however, if a retest was necessary.

The titer of each serum was obtained by making a preliminary 3-tube rough test and a final 5-tube fine test. The fine test was dispensed with for a very low titered serum (see table 2). As hemolysin titers usually vary in the rabbits from less than 10 to 5000 units/ml, the rough test was carried out routinely with log serum values of 2, 2.7 and 3.4, which equal 1, 0.2 and 0.04 ml of the 1:100 serum dilution and to which are added,

TABLE 2.—*[Log $y/(1-y)$]+1 values of the 3-tube rough test and the midpoint log value and dilution needed to set up the 5-tube fine test for titrating serums in 50% units.*

Reading from 18 3-tube rough tests at log values of			Data for the midpoint tubes of 19 5-tube fine tests		
2	2.7	3.4	Log value necessary	Dilution	Amount in ml§
Possible [log $y/(1-y)$]+1 Klett-Summerson (=KS)(added scale) reading					
No hem*	No hem	No hem			
0.3	No hem	No hem	1.4†	1:1(=und.)	0.04
0.6	No hem	No hem	1.82‖	1:1(=und.)	0.015‖
1.0	No hem	No hem	2.0	1:10	0.1
1.4	No hem	No hem	2.22‖	1:100	0.6‖
1.8	No hem	No hem	2.4	1:100	0.4
2.1	0.3	No hem	2.52‖	1:100	0.3‖
>2.1	0.6	No hem	2.6	1:100	0.25
>2.1	1.0	No hem	2.7	1:100	0.2
>2.1	1.4	No hem	2.82	1:100	0.15
>2.1	1.8	No hem	2.9	1:100	0.125
>2.1	2.1	No hem	3.1	1:100	0.08
>2.1	>2.1	0.3	3.22	1:100	0.06
>2.1	>2.1	0.6	3.3	1:100	0.05
>2.1	>2.1	1.0	3.4	1:100	0.04
>2.1	>2.1	1.4	3.52	1:100	0.03
>2.1	>2.1	1.8	3.6	1:100	0.025
>2.1	>2.1	2.1‡			

* If the KS (regular scale) reading is <20, the titer of the hemolysin can be considered approximately 10 units without carrying out any fine test. Hem, hemolysis.

† Log values of 1, 1.22, 1.4, 1.6, 1.82 are used here. For all subsequent fine tests, skip 0.1 log values instead of 0.2.

‡ Make another rough test with log values of 3.7, 4.3 and 5.0 using a 1:1000 dilution.

§ These amounts are equalized to 1 ml with VB to which a small amount of low titered normal serum is added. For convenience the VB is added first.

‖ To pipette the even amounts of 0.6, 0.3 and 0.15 ml, the log values for graphing have to be adjusted as shown here.

respectively, 0, 0.8 and 0.94 ml of VB. The VB used to equalize the serum amounts to 1 ml here and in the fine tests was modified by adding 1 part of pooled normal rabbit serums of low titer (<10 units per ml) to 200 parts of VB. This addition was found necessary because the VB and serum were routinely added to the tubes the day before the tests were actually completed and the ensuing high dilution often attained caused an overnight loss in titer when regular VB was used.

The results of each rough test were used to plan a 5-tube fine test in the manner shown in table 2. As may be seen in the next to the last column, almost any 5-tube fine test can be made with either undiluted serum or the 1:100 dilution which was made for the rough test. These two dilutions were always used, therefore, in preference to any others. The only tests that cannot be made with one of these two dilutions are those fine tests extending just under and over a log value of 2, because the serum can not occupy more than a volume of 1 ml, or extending over a log value of 4 because accurate pipetting of such small amounts can not be done rapidly. In the first case, a 1:10 dilution has to be made: in the second case, a 1:1000 dilution is necessary. A protocol of a typical 5-tube test is shown in table 3.

The 5-tube test shown in table 3 is the same one that is used for the weekly complement titration except that varying amounts of a 1:100 dilution of complement and 4 50% units of hemolysin are used. Then, if the 50% unit after graphing gives a log value of 2.3, viz., an arithmetic value of 200 50% units per ml, the complement is diluted 1:100 for testing the antiserums, since 2 ml of such a dilution contains four 50% units.

In computing the von Krogh (1916) equations for each test, we earlier used a table for the conversion of the Klett-Summerson readings to log $y/(1-y)$ values where y is the degree of hemolysis expressed as a fraction. Considerable time has been saved by two simple expedients: (1) adding 1 to the log $y/(1-y)$ values so that no minus values occur (cf line 5 and line 6 in table 3) and (2) superimposing a scale of the $[\log y/(1-y)]+1$ values on the regular Klett-Summerson scale which can be read directly.

The scale of $[\log y/(1-y)]+1$ is shown without decimals in figure 3 and is read as follows: At $y=50$ or the 50% point (150 on the regular KS scale), the $[\log y/(1-y)]+1$ scale is 1.00 as shown by 10 in the top dotted figures and zero in the bottom row. At $y=80$ (240 on the regular KS scale), it is 1.60 as shown by 16 in the top dotted figures and zero in the bottom row. At $y=20$ (60 on the KS scale), the $[\log y/(1-y)]+1=-0.6+1.0=0.4$ shown by 4 on the top scale and zero below.

To control daily and long-term variations in the titrations, two 5-tube fine tests of a hemolysin of known strength were set up, one at the beginning and the other at the end of the tests on each titration day. For example, a 1:100 dilution of a stock antiserum was made and titrated at the beginning and end of the rough and fine tests for each of 41 consecutive weeks. The resultant 164 titrations gave a mean log value of 3.7043 ± 0.0036. Sixty percent of the titrations deviated from this mean by less than 0.7% (log of titer between 3.675 and 3.725) and the greatest deviation (log of titer at 3.575 or at 3.825) was 3.4% in 2.5% or 4 of the titrations. The deviations found in this specific serum were random and they did not appreciably affect the semilogarithmic graphs of individual rabbits as they were equalized when theoretical lines were fitted to the graphs. The two sheep from which the red cells were obtained gave indistinguishable results.

Measures of antibody production.—We have

TABLE 3.—*A 5-tube fine test to obtain 50% hemolytic units for a serum for which the first tube of the 3-tube rough test read 1.5 to 1.6 (added scale) on the KS (see table 2) dial.**

Wassermann tubes	1	2	3	4	5
VB (in ml)	0.2	0.4	0.5	0.6	0.7
1. Log of dilution of antiserum (=x)	2.1	2.22	2.3	2.4	2.52
2. Ml of x (1:100 dilution)	0.8	0.6	0.5	0.4	0.3
Standardized red cells (ml)	2.0	2.0	2.0	2.0	2.0
Mix and let stand for 10 minutes or more.					
Standardized complement (ml)	2.0	2.0	2.0	2.0	2.0

Mix. Incubate at 37 C for ½ hour. Centrifuge at 1500 r.p.m. for 6 to 8 minutes.
Pour supernatant from each tube into a cuvette. Obtain KS readings for the 5 tubes. These could be as shown in line 3. Conversion of line 3 into equivalent values are shown in lines 4, 5 and 6.

3. KS readings (regular scale)	222	186	150	132	72
4. Percent hemolysin (one third of KS) =y	74	62	50	44	24
5. Log $y/(1-y)$	+0.45	+0.18	0	−0.2	−0.5
6. $[\text{Log } y(1-y)]+1$ (added scale on KS)*	1.45	1.18	0.98	0.8	0.5

* For the equivalence of the regular and added scale on the KS dial, see text. The figures, given here, are written for convenience without decimals in figure 3.

shown earlier that the rise and fall of hemolysin in individual rabbits closely follow a discontinuous curve, each segment of which is described by the integral

$$A_t = A_0 e^{k_1 t}$$

in which A_0 and A_t represent antibody titers at time 0 and a particular time. From such a curve for the hemolysin titers from each rabbit, the following measurements have been made which best characterize hemolysin formation as described by us earlier (1952 and 1954a): (1) log peak titer, (2) arithmetic peak titer, (3) the induction period in days, i.e., the interval after injection of red cells to the beginning of the rise in serum antibody, (4) the length in days of antibody rise to peak titer which begins at the end of the induction period, (5) the rate (k_1) of antibody change during the first and most rapid rise of antibody in the serum and (6) the average rate (derived k_1) for the entire initial rise from the end of the induction period to the time of peak titer when the rise consists of more than 1 phase. The k_1 rates were adjusted if the preimmunization titer was more than 10 units by drawing a line at the end of the induction period from 10 units to peak titer (or to the titer at the end of the first phase) minus the preimmunization titer. The adjusted line was appreciably different from the original line in those relatively few cases in which the preimmunization titer was 100 units or more. Thus, a k_1 rate of 0.4 would change to 0.9 after adjustment when the titer rose from 100 units to 500 units in 4 days and would change from 0.95 to 1.46 when the titer rose to 5000 units. Fifty percent of the rabbits had a preimmunization titer of 20 units or less and 5% had a titer of 100 to 180 units per ml. The rest were intermediate.

The rate constants for antibody change (k_1) were computed from the usual equation

$$k_1 = \frac{\ln A_t - \ln A_0}{t}$$

where t is the time in days. Since k_1 is the tangent of the angle subtended by the curve of the rise in antibody and the time axis, it can be determined graphically. The angle is first measured with a protractor. The value of k_1 is then obtained by multiplying the usual tangent value read from a table of tangents by 0.23. This correction is necessary because we routinely use semilogarithmic paper on which 1 log of titer on the ordinate axis is 10 times 1 day on the abscissa axis as shown in figures 4, 5 and 6. Therefore, the tangent of the angle read by the protractor is multiplied by 0.1 to accommodate for the difference in the ordinate and abscissa units and by 2.3 to convert $\log_{10}$ to ln. For example, an angle reading 72.5° with a tangent of 3.17 gives a k_1 value of 0.73.

EXPERIMENTAL RESULTS

The experiments reported in this paper have been in progress for several years. During this time, the antigen, time and method of injection, titration procedures, graphing, etc., have remained unchanged. The standardized amount of sheep red cells, which is equivalent to 1.25 ml of a 1% suspension, was injected once into a left ear vein of each rabbit per kg weight of rabbit 2 days after irradiation, shielding from X rays, or the various control surgical procedures. In comparing the antibody results in the various series of animals, we have used mean peak titer as the best single indicator of the amount of antibody formed and the 4 other measures, listed previously, as measures of the rate of the immune process. In the case of peak titers, we have used the arithmetic means to compare the various series and log titer means for all statistical constants because they give a more normal distribution (see W. H. and L. G. Taliaferro, 1954a). For the other measures, the arithmetic values are valid for both purposes.

Daily arithmetic titers for 28 days were averaged from the smoothed fitted semilogarithmic plots of all individual rabbits in a given series to obtain the mean hemolysin titers plotted semilogarithmically in figures 4 through 6. Because peak titers of the individual rabbits rarely fell on the same day in any particular series, the average arithmetic peak titer obtained for a given series is always lower in the figures than the mean arithmetic peak titer in tables 4, 5 and 6. Similar differences in the other measures occur between the average values in the figures and the mean values in the tables. For example, the mean induction period in series A6 was

8.7 ± 1.3 days in table 5, but ended on the 5th day in figure 5. This seeming discrepancy is accounted for by the fact that the induction period was 5 days in 2 rabbits but was 10.8, 11 and 14 days in 3 others.

1. Irradiation of the total body except for the lead-shielded spleen 2 days before the intravenous injection of red cells.— Irradiation of the total body except for the spleen with controls was carried out under several conditions as shown in table 4, series A, B and C 1 through 5. The rabbits in series 1 were obtained from Sharp whereas those in series 2 through 5 were obtained from Swift. Series 2 and 3 were done 3 years apart. Series 4 which is graphed in the next section involving splenectomy received 500 r and the remaining series received 700 r. The rough shielding in series A5 was done by placing 2-inch half cylinders of lead over the rabbit, whereas surgical procedures were used in the other 4 series and their controls. The X-ray constants were the same for series 2 and 5 but varied for the other series (table 1).

Before considering the effects of spleen-shielding, the two characteristic responses elicited in the nonirradiated and irradiated controls will be briefly described. These differed radically from each other as reported by us previously (see especially 1950 and 1954a). As may be seen in table 4, the 5 nonirradiated series, after a short induction period (3 to 4.7 days), reached a peak titer of 2610 to 4740 units at comparatively rapid rates of rise (1.01 to 1.62) during 4.2 to 5.2 days, whereas the 5 irradiated series had a much longer induction period (7.9 to 19.1 days), a markedly depressed peak titer (40 to 420 units), reached at much lower rates ($k_1 = 0.18$ to 0.57) during a longer antibody rise to peak titer (9.2 to 12 days).

The means for the 5 series within each control group were remarkably similar. Thus, the greatest difference in

TABLE 4.—*Hemolysin formation* in (A) 5 series of rabbits whose spleens were shielded from X rays† 2 days before one intravenous injection of 1.25 ml of 1% sheep red cells along with (B) nonirradiated controls and (C) total body irradiated controls.*

Series*	Treatment†	Peak titer			Rate (k_1) of accumulation		Length in days of:		Number of rabbits
		Log	Geometric $\times 10^3$	Arithmetic $\times 10^3$	First and most rapid rate	Average rate to peak titer	Induction period	Antibody rise to peak	
				A. Spleen-shielded: X rays to rest of body					
A1	700 r Op	2.63 ± .30Z	0.43	1.06	0.74 ± .16Z	0.67 ± .17	6.8 ± 1.6	6.2 ± 2.3	7
A2	700 r Op	1.53 ± .22X	0.03	0.07	0.18 ± .06X	0.18 ± .06X	5.3 ± 1.2z	12.1 ± 2.7Z	3 (3)
A3	700 r Op	2.04 ± .10X	0.25	0.14	0.37 ± .07X	0.37 ± .07Y	7.6 ± 1.3Yz	7.9 ± 1.4Z	8
A4	500 r Op	2.87 ± .16y	0.69	1.67	0.58 ± .06X	0.54 ± .05Xz	4.2 ± 0.6x	7.3 ± 0.4Yz	15
A5	700 r R	2.95 ± .20Zx	0.89	1.44	1.06 ± .16Zx	0.97 ± .14x	7.9 ± 0.9Xz	5.0 ± 0.7	9
				B. Completely shielded from X rays (controls)					
B1	OP	3.30 ± .11	2.00	2.61	1.25 ± .12	1.01 ± .13	3.4 ± .15	5.2 ± .45	12
B2	OP	3.23 ± .18	1.70	2.81	1.62 ± .27	1.26 ± .22	4.7 ± .39	4.2 ± .76	9
B3	OP	3.31 ± .11	2.04	2.62	1.48 ± .26	1.34 ± .25	3.7 ± .38	4.9 ± .52	10
B4	OP	3.26 ± .24	1.82	3.05	1.04 ± .11	1.02 ± .12	3.0 ± .42	4.9 ± .09	8
B5	R	3.48 ± .12	3.02	4.74	1.45 ± .10	1.18 ± .09	3.2 ± .23	4.8 ± .46	17
				C. X rays to total body (controls)					
C1	700 r OP	2.06 ± .21	0.11	0.42	0.57 ± .11	0.42 ± .11	7.9 ± 1.2	9.3 ± 2.6	7 (3)
C2	700 r OP	1.84 ± .20	0.07	0.14	0.31 ± .10	0.18 ± .02	14.5 ± 3.0	12.0 ± 1.7	6 (2)
C3	700 r OP	1.84 ± .16	0.07	0.11	0.46 ± .14	0.29 ± .07	12.4 ± 1.5	10.9 ± 2.7	5 (3)
C4	500 r OP	2.03 ± .18	0.11	0.19	0.42 ± .12	0.20 ± .04	10.3 ± 0.6	11.2 ± 1.7	7 (2)
C5	700 r R	1.53 ± .12	0.03	0.04	0.22 ± .06	0.22 ± .06	19.1 ± 4.6	9.2 ± 2.0	3 (4)

* Series A1, B1 and C1 were Sharp rabbits: All others were Swift rabbits.
All values of the hemolysin response are means ± standard errors.
Data from individual rabbits were obtained from fitted curves. Each mean peak titer was calculated from the total number of rabbits in each series: other values from rabbits which elicited an antibody response. The number of rabbits not eliciting a response is indicated by parentheses. The significance of the differences between a spleen-shielded series and its corresponding control series are indicated by the following probabilities:

	$P \leq 0.001$	$P = 0.002$ to 0.01	$P = 0.02$ to 0.05
Between series A and B	X	Y	Z
Between series A and C	x	y	z

† Op, operative technique; R, rough shielding.

log peak titer occurred between B2 and B5 (0.25 ± 0.22) in the 5 nonirradiated groups and between C1 and C2 (0.22 ± 0.29) in the 5 irradiated groups. There were only 3 statistically significant differences in series B or C between the highest and lowest mean log peak titers and the other 4 measures in table 4. These were for the k_1 first phase rate between C1 and C5 (0.35 ± 0.125) and for the induction period between B2 and B4 (1.7 ± 0.57) days), and between C1 and C5 (11.2 ± 4.75 days). The similarity for 28 days between 4 of the 5 control series of the nonirradiated groups and of the irradiated groups are well illustrated in the graphs of figure 4. These results strongly indicate that the rabbits from the two sources possessed about the same antibody-forming capacity.

A comparison of the mean arithmetic peak titers of the spleen-shielded series (A1 through A5) with the totally shielded controls (C1 through C5) indicates marked differences in the effectiveness of spleen-shielding. Figures 4A and B illustrate the extent of the protection afforded by spleen-shielding in 4 of the 5 series as compared to the nonirradiated and irradiated controls. A suitable criterion of this effect can be obtained by subtracting the arithmetic mean of each irradiated series from each corresponding nonirradiated and shielded series. Using this measure, the protection was 29% for Sharp rabbits spleen-shielded from 700 r (A1) but was nil (A2) or 1.2% (A3) for the Swift rabbits. The highest protection (52%) was obtained with Swift rabbits spleen-shielded from 500 r (A4). The average protection for these 4 series was 28%. It is interesting that the rough shielding in series A5 afforded a protection of 30% which was within the range of the surgically

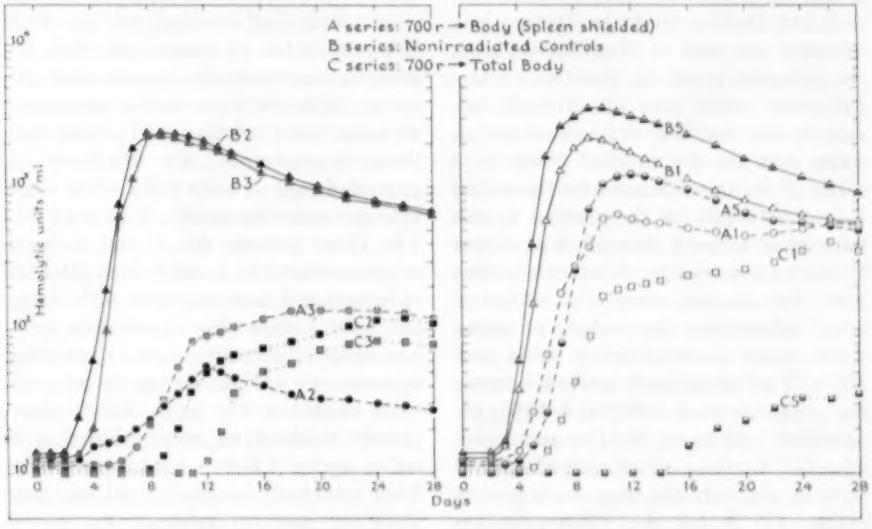

FIG. 4.—Semilogarithmic plots of mean hemolysin titers for 28 days in 4 series of spleen-shielded rabbits along with nonirradiated and total body irradiated controls. Red cells were injected intravenously 2 days later.

Note that shielding the spleen in the 4 series resulted in various degrees of partial protection (cf series A with control series B and C). More protection occurred in series A1 and A5 (right hand figure) than in series A2 and A3 (left hand figure).

shielded groups. Rough shielding, however, gave equivocal results because, on the one hand, it shielded some bone marrow and a part of the mesenteric lymph nodes while, on the other hand, it permitted a substantial percentage of the X-ray dose to reach the spleen by X-ray scattering, as reported by Wang (1953). Series A5 will, therefore, be omitted in the following analysis.

The mean log peak titer also showed the protective trend and is more suitable for statistical analysis than the arithmetic mean, as pointed out earlier. The statistical significance of the differences between each shielded series and its corresponding nonirradiated and irradiated series is shown in table 4. The mean log peak titer was consistently lower in group A than in group B and the differences in all but series 4 were statistically significant. Using Student's method of paired comparisons for all 4 series, the differences gave a probability of 0.026. Only a slight protection was afforded the rate of accumulation. As compared to group B, there was a significantly lower rate of antibody accumulation for the first phase in all series and for the derived phase in 3 series. *P* for the difference for the initial k_1 was 0.04 and for the derived k_1 was 0.03 when series 1 through 4 of A and B were compared by Student's paired test. Percentages were also obtained after subtracting the values of series C1–4 from those of series A1–4 and B1–4. The initial and derived k_1 rates for group A were 3.3% and 19%, respectively, of group B. The protection afforded to the induction period and time of antibody rise was much greater (table 4). *P* for the differences between the 4 series in groups A and B by Student's paired test were 0.09 and 0.1, respectively.

In general, therefore, spleen-shielding as compared to the nonirradiated controls almost completely protected the induction period but only allowed about 28% of the expected antibody to be formed at a much lower rate of rise within an approximately normal length of time (antibody rise to peak titer).

That the protection afforded by spleen-shielding was not marked, especially with respect to peak titer and the rates of antibody rise, is shown by the lack of statistically significant differences between the spleen-shielded series and the irradiated controls and the number of significant differences between them and the nonirradiated controls. Thus, there were no consistent differences between groups A and C except in series 4 which gave the highest percent protection (table 4).

2. Effect of the lead-shielded spleen when left in the irradiated body.—Jacobson et al (1952) found that rabbits irradiated with 800 r or 500 r retained the ability to form antisheep hemolysin when their lead-shielded spleens were left in situ for 24 hours and then removed, even though antigen was injected 24 hours after splenectomy and 48 hours after irradiation. In repeating these experiments, we irradiated 3 groups of rabbits with 500 r while their spleens were surgically lead-shielded. The three groups, A6, 7 and 8, were splenectomized 1, 3 and 5 days after irradiation and were injected with sheep red cells 2 days after irradiation or 1 day before, 1 day after and 3 days after splenectomy (table 5). Operative controls consisted of: series B6–8, completely shielded or nonirradiated animals; series C6–8, animals receiving 500 r total body irradiation without any shielding and no splenectomy; series D6–8, animals receiving 500 r total body irradiation with splenectomy 1 day after irradiation but no spleen-shielding; and series E6, 7 and 8, nonirradiated animals splenectomized 1

TABLE 5.—*Hemolysin formation* (A) in 3 series of rabbits whose spleens were shielded from 500 r and were removed 1, 3 or 5 days later along with (B) nonirradiated controls, (C) totally irradiated controls, (D) totally irradiated splenectomized controls and (E) nonirradiated splenectomized controls. The red cells (1.25 ml of 1% per kg rabbit) were injected intravenously 2 days after irradiation.†*

Series*	Treatment: X rays	Treatment: Day of splenectomy after X rays†	Peak titer: Log	Peak titer: Geometric $\times 10^8$	Peak titer: Arithmetic $\times 10^8$	Rate (k_1) of accumulation: First and most rapid rate	Rate (k_1) of accumulation: Average rate to peak titer	Length in days of: Induction period	Length in days of: Antibody rise to peak	Number of rabbits
A. Spleen-shielded: X rays to rest of body and splenectomized										
A6	500 r	1	2.02±.17X	0.10	0.21	0.40±.10X	0.35±.09X	8.7±1.3	7.9±1.2	7 (1)
A7	500 r	3	2.28±.12Y	0.19	0.24	0.51±.10Y	0.49±.11Yz	4.9±0.6z	8.3±2.1	7 (1)
A8	500 r	5	2.07±.11X	0.12	0.14	0.41±.14Y	0.39±.14Y	5.3±1.1y	7.5±1.2	8
B. Completely shielded from X rays: not splenectomized (controls)										
B6-8			3.26±.24	1.82	3.05	1.04±.11	1.02±.12	3.0±0.4	4.9±0.9	8
C. X rays to total body: not splenectomized (controls)										
C6-8	500 r		2.03±.18	0.11	0.19	0.42±.12	0.20±.04	10.3±0.6	11.2±1.7	7 (2)
D. X rays to total body: splenectomized (controls)										
D6-8	500 r	1	2.08±.21	0.12	0.23	0.46±.02	0.27±.02	15.7±2.7	11.5±2.4	4 (3)
E. No X rays: splenectomized (controls)										
E6		†	2.60±.15z	0.40	0.59	0.84±.09y	0.64±.08z	4.1±0.4y	8.5±2.1	10
E7		†	2.68±.26	0.48	0.92	0.95±.20	0.90±.14y	5.4±0.6	5.4±1.4	8
E8		†	2.89±.13x	0.78	1.00	1.10±.10x	0.91±.10y	4.7±0.6	5.6±1.1	8

* All rabbits were Swift rabbits. See table 4 for other explanations. The capital letters represent statistically significant differences as indicated in table 4 between A and B and the small letters between A and C. Series B and C are identical to series B4 and C4 in table 4. The small letters in series E6, 7 and 8 represent statistically significant differences from series A6, 7 and 8, respectively.

† The splenectomy was performed in nonirradiated groups E6 through E8 at the same intervals as in irradiated groups A6 through A8, i.e., 1 day before, 1 day after and 3 days after the injection of red cells.

day before, 2 days after and 3 days after receiving sheep red cells, respectively. In addition, series A4 in table 4 which was tested at about the same time shows the effect of lead-shielding without splenectomy. Antibody curves based on the daily mean antibody titers of each series are shown in figure 5.

The main purpose of this experiment was to ascertain the effect of leaving the spleen in the animal after it had been shielded during otherwise total body irradiation. Before considering this main question, certain relationships should be pointed out among the various controls. Examination of the data in table 5 and figure 5 indicate the following. The effect of splenectomy alone in decreasing mean log peak titer was practically the same whether the operation was done 1 day before, 1 day after or 3 days after the injection of antigen. Thus, the greatest difference was 0.29 ±0.20 log units between series E6 and 8. The average mean arithmetic peak titer of series E6, 7 and 8 was 837 units /ml or 27% of the nonirradiated controls (series B6–8). These results are in accord with our earlier studies (W. H. and L. G. Taliaferro, 1950) except that splenectomized animals receiving this amount of antigen in our earlier work only reached a mean peak of 11% of the intact controls. It is of interest that splenectomy alone resulted in almost the same mean arithmetic peak titer as occurred in spleen-shielded animals subjected to 500 r irradiation (cf E6, 7 and 8 in table 5 with A4 in table 4 and graphs of these 4 series in fig. 5). The suppressive effect of irradiation and splenectomy were partially additive. Thus, as shown by series C, D and E in table 5 and figure 5, total body irradiation was more effective than splenectomy alone, but the two together gave the most marked suppression.

Lead-shielding of the exteriorized spleen while the rest of the body was irradiated with 500 r resulted in the high-

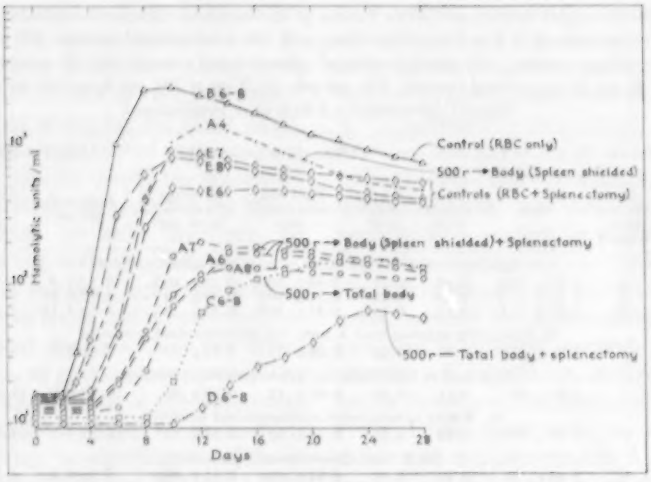

FIG. 5.—Semilogarithmic plots of mean hemolysin titers for 28 days following one intravenous injection of sheep cells in series A6 through A8 which were spleen-shielded (rest of body given 500 r) 2 days previously and from which the spleen was removed 1, 3 and 5 days after spleen-shielding, along with rabbits which were spleen-shielded (A4), not irradiated (B6-8), totally irradiated (C6–8), totally irradiated and splenectomized 1 day later (D6–8), and nonirradiated and splenectomized (E6 through E8) at the same time intervals as were A6, A7 and A8, respectively.

Note that leaving the shielded spleen 1 to 5 days in rabbits otherwise irradiated with 500 r (A6, 7 and 8) shortened the induction period but did not appreciably overcome the suppression of antibody formation due to total irradiation (C6–8) as far as peak titer and rate of rise were concerned.

est percentage protection obtained in a single series, i.e., 52% in series A4. The protection afforded by lead-shielding was practically extinguished by splenectomy whether the shielded spleen was allowed to remain in the body 1, 3 or 5 days after irradiation (i.e., 1 day befor or 1 and 3 days after antigen injection). Thus, the greatest difference in log peak titer between series C6-8 and A6, 7 and 8 was 0.25 ± 0.22 log units in series A7. Moreover, the titers in series A6, 7 and 8 were remarkably alike. The greatest difference occurred between series A6 and 7 and was 0.26 ± 0.21 log units. The difference between series A7 and 8 was 100 arithmetic units. These results are contrary to the conclusions of Jacobson et al "that the capacity to form antibodies to an injected antigen is retained in rabbits exposed to 800 r or 500 r total body irradiation if the spleen is shielded during irradiation, left intact in the circulation twenty-four hours after irradiation, and then removed surgically even though the antigen is given twenty-four hours after irradiation." Our spleen-shielded splenectomized rabbits, however, had a significantly shorter induction period when splenectomy was delayed 3 or 5 days. Whereas the animals which received total body irradiation and formed some antibody showed a mean induction period of 10.3 ± 0.6 days (series C6–8 in table 5), series A6, which retained the previously shielded spleen for 1 day showed an induction period of 8.7 ± 1.3 days, series A7 which retained the spleen 3 days had an induction period of 4.9 ± 0.6 days, and series A8 which retained the spleen 5 days had an induction period of 5.3 ± 1.1 days. Except for series A6 these differences were

highly significant. They are illustrated in figure 5.

3. Irradiation of the spleen with the body lead-shielded 2 days before the intravenous injection of red cells.—When the spleen alone was administered 500 r to 1400 r, which is a range of approximately 1 to 3 ED_{100} for antibody suppression when given to the whole body (W. H. and L. G. Taliaferro, 1954a), there was essentially a normal antibody response as compared to nonirradiated rabbits. With these doses, higher titers were reached after 1400 r than after 700 r (fig. 6), but examination of series A14 through 17 in table 6 indicates that not a single measure of log peak titer or of the rate of the immune response showed a significant difference from the nonirradiated controls (series B9–17).

In marked contrast, 5 series received from 2000 r to 10,000 r, which is a range of 4 to 20 ED_{100} for antibody suppression when given to the whole body. These series attained higher than normal antibody curves (fig. 6) and significantly higher mean log peak titers (table 6). The above normal excess in peak titer, when these 5 series were compared to the controls by Student's paired test, was 0.41 log titer, with $P < 0.001$. Administration of 10,000 r to the spleen alone (A9) resulted in a mean peak titer of about 2.5 times the control series. This result represented a true stimulation of the antibody-producing

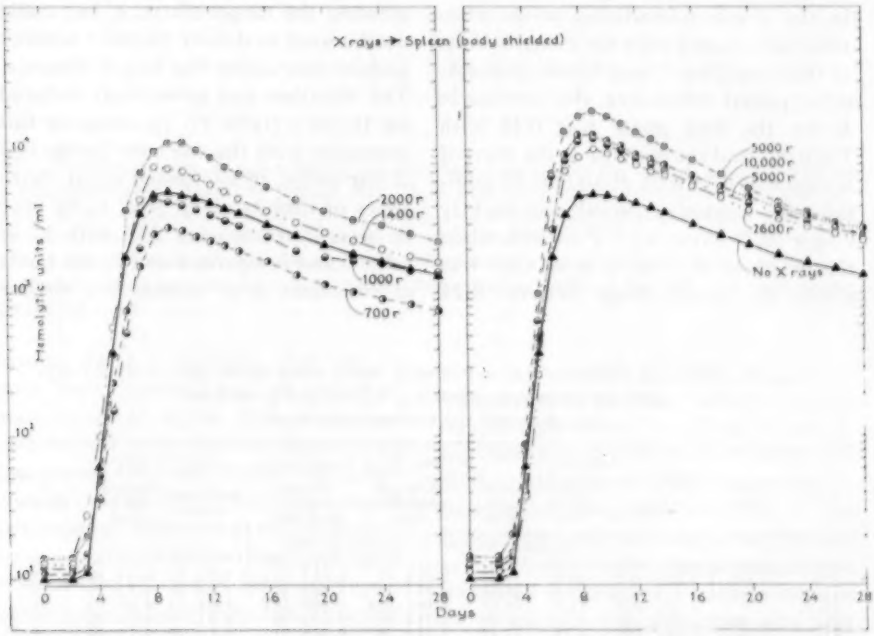

FIG. 6.—Semilogarithmic plots of mean hemolysin titers for 28 days after one intravenous injection of red cells in (A) 8 series of rabbits whose spleens were irradiated with various doses of X rays 2 days before, along with (B) a nonirradiated control series which is shown for comparative purposes in both parts of the graph (solid line and triangles).

Note that the stimulation following the larger doses of X rays to the spleen is evident by an excess of hemolysin throughout the antibody curve.

mechanism because the rate of accumulation (derived k_1) was significantly increased ($P=0.04$) while the length of the initial rise to peak remained unchanged as compared to the control nonirradiated series. Doses of 5000 r to 2 series of rabbits (A10 and A11) were equally effective. If the dosage had been raised sufficiently beyond 10,000 r, it would be expected that the spleen would be injured and antibody production suppressed.

In considering the rate of accumulation of antibody, it can be seen in table 6 that the rates for all doses showing enhancement (2000 r to 10,000 r) were consistently larger than the corresponding control rates. The rates of antibody rise were significantly faster in the 5 spleen-irradiated series when they were paired with the controls done at the same time. Using Student's method of paired differences, the increase in k_1 for the first phase was 0.46 with $P<0.001$ and the increase in the derived k_1 rate was 0.34 with $P=0.006$. In addition, the induction period was slightly longer (0.39 days), with $P=0.004$, while the length of the rise to peak titer was within the normal range (actually 0.24 days shorter, with $P=0.4$).

These results indicate that antibody formation in the rabbits whose spleens alone were irradiated with 2000 r to 10,000 r was only delayed by 0.4 days and that the enhancement was a true stimulation in that more antibody was formed at a faster rate in the normal length of time than in the nonirradiated controls. A scatter diagram of the individual log peak titers and a dosage response curve of mean log titer against log X-ray dosage (fig. 7) indicate that the degree of stimulation increased with dosage and reached an asymptotic value at about 5000 r.

As shown in table 1, the same constants in general were used from 500 r through 5000 r (11 through 17) by decreasing the target distance, but could not be used to deliver 10,000 r without unduly shortening the target distance. The filtration was accordingly reduced for 10,000 r (table 1). To integrate this procedure with the one used for the rest of the series, it was tested on an extra series of rabbits given 5000 r. As may be seen by comparing A10 with 11 in table 6 and in figures 6 and 7, the 2 sets of constants gave statistically similar

TABLE 6.—*Hemolysin formation* (A) in 9 series of rabbits whose spleens were X-rayed 2 days before one intravenous injection of 1.25 ml of 1% sheep red cells along with (B) nonirradiated controls.*

Series	Treatment	Peak titer			Rate (k_1) of accumulation		Length in days of:		Number of rabbits
		Log	Geometric $\times 10^3$	Arithmetic $\times 10^3$	First and most rapid rate	Average rate to peak titer	Induction period	Antibody rise to peak	
		A. Spleen X-rayed; rest of body shielded							
A9	10,000 r	4.09 ± .09X	12.3	14.91	2.13 ± .13Y	1.91 ± .19Z	3.9 ± .37	3.8 ± .46	9
A10	5,000 r†	4.00 ± .16Y	10.0	15.46	2.01 ± .17	1.64 ± .21	3.8 ± .17	4.6 ± .48	9
A11	5,000 r‡	4.19 ± .12X	15.5	19.67	2.33 ± .13X	1.80 ± .19	3.7 ± .18	4.5 ± .54	9
A9–11		4.09 ± .06X	12.3	16.67	2.16 ± .09X	1.80 ± .11Y	3.8 ± .14	4.3 ± .29	27
A12	2,600 r	3.91 ± .16Z	8.13	11.05	1.74 ± .07	1.63 ± .06	4.0 ± .18	4.0 ± .29	9
A13	2,000 r	3.94 ± .18Z	8.71	13.41	2.13 ± .28	1.52 ± .21	3.7 ± .28	4.7 ± .58	9
A14	1,400 r	3.68 ± .12	4.79	8.49	1.46 ± .13	1.31 ± .14	3.6 ± .40	4.5 ± .46	20
A15	1,000 r	3.60 ± .12	3.99	4.73	1.21 ± .09	1.21 ± .09	4.1 ± .21	3.3 ± .31	6
A16	700 r	3.41 ± .10	2.58	3.37	1.29 ± .11	1.10 ± .12	3.7 ± .32	4.3 ± .43	12
A17	500 r	3.66 ± .23	8.20	6.09	1.89 ± .15	1.27 ± .15	3.6 ± .31	5.3 ± .50	6
		B. Completely shielded from X rays (controls)							
B9–17		3.59 ± .06	3.90	5.58	1.68 ± .08	1.41 ± .09	3.5 ± .13	4.5 ± .28	34

* All rabbits were Swift rabbits. See table 4 for other explanations.
† Same X-ray constants as for 10,000 r (see table 1).
‡ Same X-ray constants as for 2600 r, etc. (see table 1).

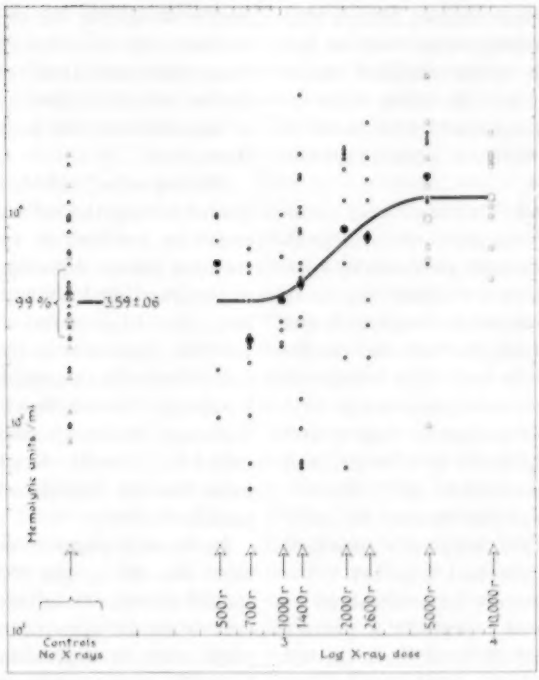

FIG. 7.—Dosage-response curve relating log peak hemolysin titer to log dose of X rays in 9 series of spleen-irradiated rabbits (A9 through A17) along with nonirradiated controls (B9–17). The different X ray constants (see table 1) and accompanying controls are indicated by open and closed circles and triangles. A large symbol indicates the mean of the series.

Note that the rabbits whose spleens received 2000 to 10,000 r had higher peak titers than the nonirradiated controls and that the rabbits whose spleens received 1400 r or less had peak titers nearer the normal range.

results. It is interesting that the log peak titer extended over an extremely wide range in series A10. The lowest and highest peak titers as shown in figure 7 were 1000 and 50,000 units. Such a high variability frequently accompanies irradiation and is reflected in the comparatively high coefficients of variation of the various series as compared to the nonirradiated controls. This tendency, however, was reversed in the rabbits receiving the highest dose to the spleen. Thus, the coefficient of variation in series A9 was only 6.6% as contrasted to 9.75% in the nonirradiated controls (series B9–17) and 36% in series A10.

DISCUSSION

Our results on the protective action on antibody formation of spleen-shielding during severe irradiation to the rest of the rabbit or of allowing the previously shielded spleen to remain in the rabbit for a day or more after the rest of the body was irradiated should be discussed in relation to the results of Jacobson and his associates who first designed these experimental methods and of Wissler et al (1953) who carried out the same type of experiments with rats. In all of our current work, a standardized suspension of approximately 1.25 ml of 1% sheep red cells

were injected intravenously 2 days after irradiation. Controls for each series (except for rough spleen-shielded series A5) involved total shielding from irradiation and total body irradiation of rabbits whose spleens were surgically exteriorized.

Jacobson and his associates concluded that the capacity to form antibodies to an injected particulate antigen is retained to a marked degree in spleen-shielded animals. As regards the amount of antibody formed, our results agree in principle with the results of Jacobson and his associates except that the protection was less in degree and was even negligible in two series of 6 and 8 rabbits (series A2 and 3). In fact, 3 of these 14 rabbits formed no antibody at all. Our surgically shielded animals, when grouped together without regard to source or amount of irradiation, showed about 28% protection, i.e., formed 28% of the adjusted amount of antibody produced by the nonirradiated group. A similar calculation for the animals given in the two papers by Jacobson et al indicated a protection of 70%. Fifty-two percent was the highest protection we obtained. These differences occurred in spite of certain similarities in the experimental procedure. For example, they used Swift rabbits to which they administered 800 r in most cases, whereas our rabbits from this source showed no protection with 700 r. They injected sheep red cells intravenously 1 or 2 days after irradiation. They used 60% more antigen than we did but this comparatively small difference is unimportant in view of our earlier studies (see W. H. and L. G. Taliaferro, 1954a). The differences, however, may be accounted for by dissimilarities in the experimental procedure which at present we cannot evaluate. For instance, in their hemolytic titrations, they used a 100% end point after incubation for an hour at 37 C and overnight in the refrigerator. Their method of titration was less exact than ours and their method of incubation would involve a larger quantity of less avid and less hemolytic antibody than ours.

Wissler et al (1953) concluded that lead-shielding the rat's spleen during irradiation resulted in antibody concentrations nearly equaling those reached in nonirradiated controls. They, therefore, also obtained more protection than we did. Parenthetically, it should be pointed out that our rabbits only formed Forssman hemolysin (Taliaferro and Talmage, 1956), whereas their rats presumably formed isophile hemolysin since the rat contains Forssman antigens in its body.

In several papers, we have emphasized the advantage of collecting data spaced closely enough to allow antibody curves to be constructed at least to peak titer in individual animals. We have also given our reasons for believing that peak titer is the best single measure for interrelating the amount of antibody formed under varying conditions. Moreover, comparisons of titers at the same time interval after the injection of antigen seldom give a true picture of the total amount of antibody formed in irradiated and nonirradiated series, when, as so frequently happens, the irradiated animals have a lengthened induction period which displaces the entire antibody curve. In such cases, statistically significant differences in titer on a given day have no meaning in reference to the total antibody formed. Therefore, to get a basis for analysis, we titrated serums at closed spaced intervals for a month or more to obtain the highest arithmetic peak titer without respect to the specific day on which it occurred (table 4). For statistical analysis, we have used log peak titers because of their more normal distribution. The rate of the immune response

was then gauged by the length in days of the induction period and of the antibody rise to peak titer in conjunction with rates of antibody rise (k_1). In measuring the degree of protection afforded by spleen-shielding, we corrected for the amount of antibody made by the totally irradiated group by subtracting the mean peak titer of the totally irradiated group from the mean peak titer of both the nonirradiated and shielded groups.

The degree of protection varied markedly. Thus, among the rabbits whose spleens were shielded from 700 r, the Sharp rabbits showed 29% protection, whereas the Swift rabbits (series A2 and 3 combined) showed no protection. This difference may be due to a more active lymphoid-macrophage system in the first group because of several concomitant infections (see Materials and Methods). The amount of X rays or the constants used and other unresolved factors also operate. Thus, among Swift rabbits under identical titration procedures, spleen-shielding from 700 r produced no protection in series A2 and A3. However, spleen-shielding from 500 r gave 52% protection in series A4 and 18% in a series (1-A1) reported by Süssdorf and Draper (1956) in which, as compared to our procedure, 8 times more antigen was injected 1 day after irradiation, i.e., at the time of maximum injury.

We also found that shielding the spleen, when it protected antibody formation, also protected the rate of formation slightly and the induction period and length of antibody rise to peak titer markedly as compared to totally irradiated rabbits. Jacobson et al found a delay in the time of peak titer and did not measure rates.

In experiments in which we surgically lead-shielded the spleen and irradiated the rest of the body with 500 r, left the spleen intact within the animal 1, 3 or 5 days and then removed it, we failed to find the degree of protection obtained by Jacobson et al (1952) but obtained results similar to those of Wissler et al when they injected rats intravenously. We found that the shielded spleen, if left in the irradiated animals 3 to 5 days, failed to increase peak titer or rates appreciably but shortened the induction period as compared to totally irradiated animals. These findings suggest that, in part at least, the factors which suppress the amount of antibody formed are different from those which cause the induction period to be lengthened. Such a separation of factors is also suggested by the results of Jaroslow and Taliaferro (1956). These workers found that rabbits given 400 r total body irradiation, which markedly reduces the power to form hemolysins, formed normal amounts of antibody when the red cells were mixed with extracts of HeLa cells or with yeast autolysate shortly before injection and were injected one day after irradiation. Although such material permitted the animal to form normal amounts of antibody at normal rates (derived k_1), the induction period remained as long as the totally irradiated controls which received antigen alone.

Wissler et al (1953) found that 500 r total body irradiation markedly depressed antibody formation in the rat following a single injection of foreign red cells administered intravenously or intraperitoneally just before, 24 hours or 48 hours after irradiation. Lead-shielding of the spleen during irradiation largely abolished this X-ray depression of antibody formation. When, however, the shielded spleen was allowed to stay in the rat 24 or 72 hours after irradiation, the protective action of the shielded spleen was noticeable in rats which received antigen intraperitoneally but not in those which received it intravenously.

It is of great interest that the lack of protection, which we found from leaving the shielded spleen in the irradiated animal for a day or so when the antigen was given intravenously, agrees with the result of Wissler et al but is opposed to their suggestions regarding the role of the spleen in the rat and rabbit. Thus, they believe that the rat differs from the rabbit following the intravenous injection of particulate antigen in that most of the antibody is formed in the spleen in the rat and in nonsplenic sites in the rabbit, but that nonsplenic sites are active in both animals after intraperitoneal injections. As a consequence, removal of the lead-shielded spleen from the rat after intravenous immunization does not protect antibody formation because the intact spleen is necessary, whereas removal of the lead-shielded spleen from the rat after intraperitoneal and from the rabbit after intravenous immunization results in nearly normal antibody production because of the protective effect of the shielded spleen on extrasplenic antibody-forming tissues. We agree with Wissler et al that the evidence to date indicates that the spleen is relatively more important in immunization with particulate antigens via the intravenous route in the rat than in the rabbit. We believe, however, that they overemphasize this difference. Moreover, they overlooked the evidence that we (1950) presented indicating that after a single injection of 1 ml of 1.25% sheep red cells/kg (approximately a 50% larger dose than they used), splenectomy reduces the titer to about 11% of the controls. In the present work with the same amount of antigen, there was a reduction to about 28%. These results indicate a large involvement of the spleen in antibody formation in the rabbit.

Both Jacobson et al for the rabbit and Wissler et al for the rat relate the protection afforded by lead-shielding the spleen to the role of the spleen in forming antibody. We believe this association in the rabbit is fortuitous. Thus, in the nonirradiated rabbit, removal of the spleen greatly reduces the antibody formed, whereas removal of the appendix does not significantly lower (Süssdorf and Draper, 1956) and occasionally increases peak titer (W. H. and L. G. Taliaferro, 1950). Nevertheless, as shown first by Jacobson et al (1950), shielding the appendix exerts just as great a protection as shielding the spleen. In fact, in the following paper by Süssdorf and Draper (1956), it will be shown to be far more effective. These data suggest that the important factor is the relative mass of lymphatic tissue which is shielded rather than the special organ shielded. In the rabbit, the appendix is much larger than the spleen while in the rat the spleen is itself relatively large. The lack of the necessity of the shielded tissue to form antibody indicates that the shielded tissue possesses some factor which induces regeneration of the antibody-forming tissue. The factor may act locally when the chief locus of antibody-formation is shielded or at a distance when other tissue is shielded. It may be cellular and /or noncellular in nature. In so far as it is noncellular (cf Jacobson, 1956), it may be akin to the substance which occurs in HeLa cells and yeast autolysate which Jaroslow and Taliaferro (1956) found would restore the capacity of X-rayed animals to form antibody.

The older literature contains reports that small amounts of X rays given locally enhance antibody formation, but the findings are difficult to evaluate at the present time (see review by W. H. and L. G. Taliaferro, 1951b). Mention, however, should be made of Manoukhine (1913), who reported that guinea pigs, after irradiation with 1 H (probably about 125 r) over the region of the spleen a day after the injection of an-

tigen, gave hemolytic titers 2 to 5 times higher than nonirradiated controls.

In our recent work, we found two types of enhancement of antibody produced in totally irradiated rabbits (W. H. Taliaferro et al, 1952, and W. H. and L. G. Taliaferro, 1954a and b). The first type occurred when antigen was injected a few hours before 300 to 700 r total body irradiation. This increase of peak titer was associated with a marked retardation of the immune process as indicated by a lengthened induction period and a slower rate of rise of serum antibody. It is not a true stimulation but appears to be the result of the activity of an injured antibody-forming mechanism that functions over a sufficiently long period and eventually produces high peak titers. The second type occurred with much lower amounts of total body irradiation (10 and 25 r but not 50 r) and was not associated with any evidence of a retardation of the immune response. Since the increase in log peak titers over the normal was statistically significant ($P \leqq 0.001$), we do not doubt its occurrence, but believe further work is necessary because of the extreme variability encountered between series tested at various times although presumably under like conditions. It is doubtful that either type of excess antibody formation involves the same mechanisms as in the true enhancement found in our present work with larger doses of X rays to the spleen alone.

The marked enhancement of antibody formation in rabbits whose spleens alone received large doses of X rays (2000 to 10,000 r) 2 days before the intravenous injection of red cells is in many ways the most remarkable finding reported in the current work. Identical mechanisms may be involved in our work and in the local stimulation of hemolysin formation reported by Graham et al (1956) when they locally irradiated the rabbit with 1000 r immediately after and again 1 week after the intradermal injection of sheep red cells. Their procedure and results, however, more nearly resemble the type obtained by us when antigen was injected shortly before severe total body irradiation since they found that the antigen had to be in the tissue at the time of irradiation.

SUMMARY AND CONCLUSIONS

Rabbit antisheep hemolysins titrated in 50% hemolytic units have been used (1) to study the protective effect on hemolysin production of lead-shielding the surgically exteriorized spleen of the rabbit receiving 500 r or 700 r X radiation 2 days before the intravenous injection of a photometrically standardized suspension of sheep red cells ($\approx$1.25 ml of 1% cells) per kg rabbit; (2) to ascertain the result of splenectomizing such rabbits 1, 3 or 5 days after X radiation; and (3) to compare the effects of lead-shielding the body while irradiating the spleen with 500 to 10,000 r 2 days before the intravenous injection of the same amount of red cells. Surgically operated control series involved exteriorizing the spleen and either shielding the rabbit completely from irradiation or totally irradiating the rabbit.

Surgical lead-shielding of the spleen from 500 r to 700 r protected hemolysin production in 4 series of rabbits from 0 to 52%. These values were obtained from peak titers of the spleen-shielded series in terms of the nonirradiated controls after subtracting from each series the peak titers of totally irradiated controls. The degree of protection varied with the source of the rabbits and the amount of irradiation. The average protection for the whole series was 28%. In addition, there was a slight protection of the rate of formation (k_1 values) and a more marked protection in the

lengths of the induction period and initial rise to peak titer.

Protection was only afforded to the induction period by leaving the shielded spleen in an otherwise irradiated rabbit 3 or 5 days. Peak titers, rates of rise (k_1) and the length of the antibody rise to peak titer were practically the same as in irradiated controls.

Rough shielding of the spleen by externally placed lead strips afforded 30% protection in one series administered 700 r.

Antibody formation was stimulated as compared to nonirradiated controls in rabbits whose spleens received large doses of X rays (2000 to 10,000 r) as indicated (after a slightly lengthened induction period) by an augmented peak titer produced at a slightly higher rate during the normal length of time.

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THE PRIMARY HEMOLYSIN RESPONSE IN RABBITS FOLLOWING SHIELDING FROM X RAYS OR X IRRADIATION OF THE SPLEEN, APPENDIX, LIVER OR HIND LEGS

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W. H. and L. G. Taliaferro (1950, 1951, 1952) have made extensive studies on the function of the spleen in hemolysin formation in the rabbit. They (1950) showed that splenectomy performed 14 days before through 4 days after injection of a single dose of sheep red cells caused a slower hemolysin rise and depressed peak titer. Appendectomy, used as an operative control procedure in their experiments, resulted in occasionally higher hemolysin titers. Jacobson et al (1950) were the first to report that lead-shielding of the spleen or appendix during 800 r body X irradiation essentially protected the antibody-forming capacity of rabbits to sheep red cells except for some delay of the response. Wissler et al (1953) extended these observations to the rat, using a dose of 500 r and the same antigen. W. H. and L. G. Taliaferro (1956) repeated the spleen-shielding experiment for the rabbit, employing more quantitative techniques for evaluating the hemolysin response, and observed less protection than did Jacobson et al (1950). In the same paper these authors reported the effect on hemolysin formation of large doses of X rays to the spleen alone. They found that above normal peak titers were attained with 2000 r or more.

The role of the liver in antibody formation is, at present, of debatable significance. A number of authors (Hektoen and Curtis, 1915; Oakley et al, 1951; and others) have concluded that the liver plays only a minor role in antibody production. Miller and Bale (1954) and Miller et al (1954) found that γ globulins are not produced normally by rat liver although the rest of the plasma proteins are formed in that organ. Thorbecke and Keuning (1953) could not demonstrate in vitro antibody formation by liver tissue of rabbits immunized with *Salmonella paratyphi* B vaccine. However, liver tissue of splenectomized rabbits vaccinated with the same antigen or of intact rabbits injected with horse serum did produce small amounts of antibody in vitro (Thorbecke and Keuning, 1956). Other workers (Müller, 1911; Ranney and London, 1951) have presented evidence that the liver may play an important role in the formation of antibodies. We (unpublished results) found that extirpation of 75% of the liver of the rabbit followed by one intravenous injection of heated sheep red cell stromata produced only a significant shortening of the initial rapid rise of antibody to peak titer. Havens et al (1956) reported, however, that the removal of 70% of the liver of rats re-

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sulted in above normal antibody production after the intravenous injection of sheep red blood cells. These authors suggest that the increase in antibody formation is a function of some specific stimulus associated with the removal of a large portion of the liver or its subsequent regeneration. Hecht and Potter (1955) have observed marked synthesis of deoxyribonucleic acid in regenerating rat liver beginning 18 hours after partial hepatectomy and reaching a maximum 24 to 30 hours postoperatively. DNA formation is preceded by an increased rate of ribonucleic acid synthesis.

The comparative effects on the hemolysin response of shielding from X rays or X-radiating a portion of the animal body preceding either local injection into that part or intravenous administration of antigen have, to our knowledge, not been reported. The following papers have some bearing on this objective. Lecomte and Fischer (1949) failed to obtain an expected elevation of the antibody titers in rabbits with high anti-horse serum precipitin levels by X radiating the hind legs. In addition, a re-evaluation of these authors' data did not show a significant decrease of serum titers. Thompson and Harrison (1954) X-radiated the heads of rabbits three days after intracorneal injection of various protein antigens without observing any effect on corneal antibody concentration. However, X radiation of the body with the head shielded eliminated antibody formation in the cornea completely. These authors believe that in the latter case either a general factor necessary for antibody production was destroyed or some inhibitory substance was formed. Smith et al (1956), using the hemolsyin response as the indicator system, effected partial body shielding of mice by using perforated lead plates covering the whole body during X irradiation. They concluded that the damage to the production mechanism of hemolysins appears to be proportional to the amount of the body irradiated. Finally, Graham et al (1956) injected sheep red cells intradermally in the hip region of rabbits and focally irradiated this area immediately and one week later with 1000 r. These workers noted above normal hemolysin titers in animals treated in this manner. Graham and Leskowitz (1956), using alum precipiated diphtheria toxoid in a similar study, found an enhanced production of circulating antitoxin and an even larger increase of antibody at the site of injection and irradiation.

The present work is a comparative study of the effects on the primary hemolsyin response in rabbits of shielding from X rays or exposing to X rays the spleen, appendix, liver or hind legs. Intact, splenectomized and splenectomized-appendectomized animals were used. A single dose of a 10% suspension of heated sheep red cell stromata/kg was injected intravenously or subcutaneously one day following extirpation and/or irradiation procedures. In all cases, serum hemolysin levels were determined colorimetrically by employing the 50% end point titration method.

With these methods we attempted to study comparatively the function of the spleen, appendix and liver in the production of hemolysins in the rabbit and to demonstrate their role in the protection of the antibody-producing mechanism from X-ray injury. We also investigated the contribution of the regional lymph nodes to the total antibody response by injecting sheep red cell stromata into the hind foot pads one day after X-irradiating or shielding from X rays the hind legs. The immune response elicited in this manner was compared to that obtained after an intravenous injection, the other variables remaining the same. A related objective in performing this

experiment was to reveal the possible protective effect on the hemolysin-producing mechanism of shielding the hind limbs during body X irradiation.

MATERIALS AND METHODS

Animals.—The rabbits were obtained from Mr. Harold Swift, Lakeside, Mich. They were free of respiratory and intestinal infections. Random selection of rabbits resulted in the use of approximately equal numbers of males and females which weighed between 1.6 and 2.8 kg with the great majority ranging between 2.0 and 2.3 kg.

Surgery.—Anesthesia consisted of ether and pentobarbital sodium for shielding procedures and ether alone for animals not receiving X rays. To ease the manipulation of the spleen or liver by reducing the size of the stomach, the rabbits were starved for 24 hours preoperatively. A midline abdominal incision was made after shaving the ventral area. All control groups except A3, A4, C3 and C4 of experiment 3 were incised. The operating time for controls was similar to that needed for the corresponding experimental groups. After pouring 3 or 4 ml of a saline solution of penicillin and streptomycin (cf W. H. and L. G. Taliaferro, 1956) into the peritoneal cavity, the incisions were closed in 2 layers with #24 cotton thread.

Splenectomy.—The spleen was excised after ligating the splenic blood vessels 2 or 3 at a time.

Appendectomy.—The caecal end of the appendix was teased from the adjacent ileum exposing the appendicular artery and vein which were ligated at the appendocaecal junction. The subsidiary ileoappendicular vessels were tied off, 2 or 3 at a time. The appendix was excised distal to a Carmalt forceps closed over the appendix immediately distal to the ligation of the appendicular artery and vein. The caecum was closed by over-sewing the clamp and was followed by a purse-string invagination of the stump.

X rays.—The X-ray constants used for the 500 r dose to the body with or without organ-shielding at the Argonne Cancer Research Hospital were as follows: 30 Ma, 250 Kv, 0.5 mm Cu and 1.0 mm Al filters which gave a half value layer of 1.43 mm Cu and a 125.5 cm target distance which resulted in about 20 r per minute. For selective organ irradiation, the target distance was changed to 73.5 cm to deliver 1400 r at approximately 54 r per minute. Thus irradiation time for both doses was about 25 minutes.

Shielding.—The spleen and appendix shields, and the body shield for spleen irradiation together with their application have been described by W. H. and L. G. Taliaferro (1956). The body shield for appendix irradiation was identical to the body shield for spleen irradiation except for a 1 cm hole in the former to permit passage of the caecal end of the appendix.

The liver and liver-exposure shields whose lead thickness was 0.6 cm or more were designed and cast by us from plaster models and molds. The liver shield (fig. 1) consisted of an open-end, semiconical base and a lid-like cover. With the animal on its right side, the surgically exteriorized liver was introduced into the base, covered with gauze wet with antibiotic saline solution and the cover applied. Insertion of the shield resulted in only about 16 cm^2 shield shadow on the underlying abdominal wall.

The liver-exposure shield (fig. 2) consisted of 2 baffle-edged halves which provided a deep depression to accommodate the liver and a small hole at the bottom of the trough for passage of the hepatic vessels. Photographic plate tests showed no X-ray scattering around the hole (cf Wang, 1953). Before the main liver (75% of the total liver mass) was introduced into the shield, the ligament extending between the main lobes, the diaphragm and the vena cava was severed. The posterior liver lobe (25% of the total mass) could not be exteriorized sufficiently without rupturing its separate, draining vessels.

The contours of the liver-exposure shield halves were suitable for both leg shielding and body shielding (legs exposed). Additional lead sections overlapped the contoured shields to complete the desired shielding.

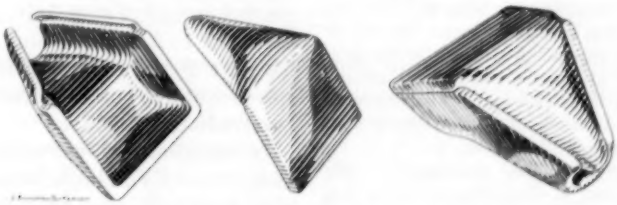

FIG. 1.—Body, lid and combined parts of the liver shield used in experiment 3. ×½.

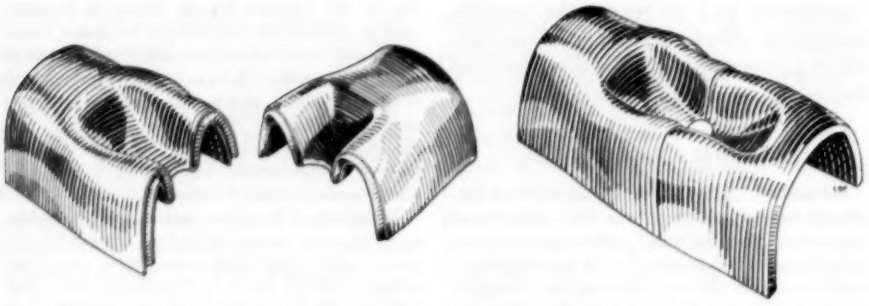

FIG. 2.—The separate and combined halves of the liver-exposure shield used in experiment 3. Two semicylindrical end shields were added to protect the rest of the rabbit. $\times \frac{1}{4}$.

Antigen.—A single dose of 1.6×10^9 heated sheep erythrocyte stromata (equivalent to 1 ml of 10% red cells) per kg was injected one day following the operative and/or shielding procedures discussed above. Irradiation was done immediately following the operative procedures. Rabbits in experiments 1, 2 and 3-A and 3-B were injected intravenously into the marginal vein of the left ear and in experiments 3-C and 3-D were injected subcutaneously with half the dose into each hind foot pad. The stromata were prepared according to the method outlined by Jaroslow and Taliaferro (1956), which consisted of gently lysing fresh sheep red cells in various strengths of phosphate-buffered saline solution. They were counted in a hemocytometer chamber with a phase contrast microscope and then autoclaved at 2 lbs pressure (approximately 103 C) for 1 hour.

Serum samples.—Following the injection of antigen, the animals were bled from the right ear every day for the first week, 3 times a week for 2 weeks and twice weekly for another week or two. Serum from each 3 ml blood sample was collected separately by centrifugation.

Titration.—The methods for hemolysin assay were the same as those described by W. H. and L. G. Taliaferro (1956). All titers are expressed in 50% hemolytic units contained in 1 ml of serum, a 50% hemolytic unit being defined as the reciprocal of that amount of serum (in ml) required to lyse one-half of the cells in the presence of 2 ml of a solution of complement containing four 50% hemolytic units. The degree of hemolysis was determined photometrically with a Klett-Summerson colorimeter.

The titers were graphed semilogarithmically against time and a discontinuous curve was fitted to the points following the reasoning presented by W. H. and L. G. Taliaferro (1950). Student's *t* test was used to compare various groups. Mean daily titers for each group are plotted in the figures to illustrate the antibody curves for each experimental and control group except 1-A5 and 1-B5.

EXPERIMENTAL RESULTS

The material presented in this paper consists of three experiments, each of which is subdivided into several parts. These parts, in turn, are made up of various experimental groups of five to ten animals each. The experiments are outlined in the first five columns of table 1.

In experiment 1-A, the spleen was shielded or irradiated. Three control groups consisted of totally irradiated, nonirradiated and splenectomized rabbits. In part B these procedures were repeated for the appendix.

In experiment 2-A and 2-B, the livers of intact and appendectomized-splenectomized rabbits, respectively, were shielded or irradiated. The two control groups of part A are given in experiment 1 (A3 and 4), and the controls for part B involved appendectomized-splenectomized totally irradiated and nonirradiated rabbits.

In experiment 3, the hind legs of intact (A and C) and splenectomized rabbits (B and D) were shielded or irradiated. While the previous two experi-

TABLE 1.—*Effect on the primary hemolysin response of heated sheep erythrocyte stromata injected 1 day following shielding from X rays or X irradiation of the spleen, appendix, liver or hind legs of rabbits after various surgical procedures. Group means are shown for six and standard errors for five response characteristics.*

Exp. and group	Treatment*			Peak titer		Length in days of:		Rate of antibody accumulation (k_1)		Number of rabbits
	Surgery	X irradiation	Route of injection	Log	Arithmetic $\times 10^3$	Induction period	Antibody rise to peak	First phase	Derived when > one phase	
Spleen study										
1-A 1	I	500r→B(S)	IV	2.92±.24	1.40	4.6±0.5	7.6±0.8	0.51±.11	0.42±.09	5
2	I	1400r→S(B)	IV	4.05±.08	12.08	3.9±0.0	5.6±0.3	2.05±.28	0.96±.12	5
3	I	500r→T	IV	1.97±.14	0.11	13.0±1.1	14.4±1.8	0.44±.08	0.16±.03	5
4	I	No X rays	IV	3.83±.07	7.14	2.6±0.1	7.0±0.4	1.47±.07	0.84±.06	5
5	S	No X rays	IV	2.39±.14	0.41	5.3±0.5	7.0±0.9	0.63±.26	0.43±.07	10
Appendix study										
1-B 1	I	500r→B(A)	IV	3.98±.21	13.58	5.5±0.2	6.5±0.2	1.39±.09	0.94±.05	5
2	I	1400r→A(B)	IV	3.88±.08	8.00	2.9±0.2	7.0±0.4	1.94±.14	0.89±.20	5
3	I	500r→T	IV			Identical with 1-A3				5
4	I	No X rays	IV			Identical with 1-A4				5
5	A	No X rays	IV	3.54±.09	4.25	2.7±0.2	8.3±0.3	1.15±.13	0.58±.05	10
Liver study—intact rabbits										
2-A 1	I	500r→B(L)	IV	2.83±.23	1.20	7.2±0.6	9.6±1.7	0.48±.16	0.34±.08	5
2	I	1400r→L(B)	IV	3.72±.16	6.77	3.1±0.1	4.8±0.3	1.09±.12	1.09±.12	5
3	I	500r→T	IV			Identical with 1-A3				5
4	I	No X rays	IV			Identical with 1-A4				5
Liver study—appendectomized splenectomized rabbits										
2-B 1	AS	500r→B(L)	IV	2.25±.34	0.45	9.8±1.0	11.6±1.4	0.78±.19	0.30±.05	5
2	AS	1400r→L(B)	IV	3.64±.20	6.23	3.5±0.2	7.3±0.8	1.03±.08	0.85±.15	5
3	AS	500r→T	IV	1.76±.16	0.08	20.8±3.5	8.8±3.2	0.50±.09	0.31±.09	5
4	AS	No X rays	IV	3.44±.29	5.88	4.2±0.4	7.6±1.9	1.15±.25	0.97±.34	5
Leg study—intravenous injection; intact rabbits										
3-A 1	None	500r→B(Lg)	IV	2.66±.09	0.51	7.0±0.3	6.8±1.6	0.64±.09	0.60±.12	5
2	None	1400r→Lg(B)	IV	3.43±.02	2.67	2.9±0.5	7.8±1.5	1.58±.38	0.76±.16	5
3	None	500r→T	IV	1.91±.09	0.09	13.4±1.1	17.4±3.8	0.14±.04	0.13±.03	5
4	None	No X rays	IV	3.21±.14	2.49	3.6±0.8	4.2±0.5	1.33±.12	1.11±.12	10
Leg study—intravenous injection; splenectomized rabbits										
3-B 1	S	500r→B(Lg)	IV	2.99±.32	2.06	6.8±1.0	7.8±1.0	1.00±.24	0.55±.05	5
2	S	1400r→Lg(B)	IV	2.89±.14	1.02	4.0±0.2	7.5±0.9	0.98±.12	0.58±.08	6
3	S	500r→T	IV	1.99±.08	0.11	15.3±1.6	12.3±2.4	0.32±.08	0.22±.07	5
4	S	No X rays	IV			Identical with 1-A5				10
Leg study—foot pad injection; intact rabbits										
3-C 1	None	500r→B(Lg)	FP	2.27±.12	0.22	4.0±0.0	20.1±1.7	0.81±.16	0.15±.02	5
2	None	1400r→Lg(B)	FP	3.26±.07	1.92	3.8±0.1	6.8±0.3	1.42±.20	0.54±.06	5
3	None	500r→T	FP	2.09±.15	0.16	10.8±1.3	11.9±2.6	0.41±.06	0.23±.00	5
4	None	No X rays	FP	3.29±.08	2.26	3.0±0.2	7.3±1.1	1.35±.13	0.76±.14	10
Leg study—foot pad injection; splenectomized rabbits										
3-D 1	S	500r→B(Lg)	FP	2.53±.18	0.44	4.9±0.8	9.6±1.0	0.36±.08	0.30±.05	5
2	S	1400r→Lg(B)	FP	3.23±.05	1.76	3.7±0.2	6.3±0.2	1.42±.15	0.74±.07	5
3	S	500r→T	FP	2.14±.15	0.18	11.8±0.9	20.0±2.7	0.26±.10	0.11±.00	5
4	S	No X rays	FP	2.97±.09	1.11	3.4±0.1	4.8±1.0	1.36±.14	1.11±.19	10

* Explanation of symbols: I = incision only; S = splenectomy; A = appendectomy ... ojection into the marginal vein of the ear; FP = injection into the hind foot pads.

500r→B(S) = 500r to the body with the spleen shielded.
1400r→S(B) = 1400r to the spleen with the rest of the body shielded.
500r→B(A) = 500r to the body with the appendix shielded.
1400r→A(B) = 1400r to the appendix with the body shielded.
500r→T = 500r to the total body.
500r→B(L) = 500r to the body with the liver shielded.
1400r→L(B) = 1400r to 75% of the liver with the rest of the body shielded.
500r→B(Lg) = 500r to the body with the hind legs shielded.
1400r→Lg(B) = 1400r to the hind legs with the rest of the body shielded.

ments involved only intravenously injected animals, this experiment included injections into the two hind foot pads (C and D) as well as intravenous injections (A and B). Totally irradiated and nonirradiated control groups consisting of intact and splenectomized animals injected by the two routes completed this experiment. (Control group A5 in experiment 1 served as control group 3–B4 in this experiment.)

In all parts of each experiment, group 1 involved shielding from X rays and group 2 irradiation of the organ studied.

All groups 3 were totally irradiated and groups 4 remained nonirradiated. Groups A5 and B5 of experiment 1 were splenectomized and appendectomized controls, respectively.

Table 1 lists the group means of six characteristics of the hemolysin response. They are (1) the arithmetic and (2) the logarithmic peak titers in 50% hemolytic units, (3) the length in days of the induction period (i.e., the period of time between antigen injection and beginning of antibody rise), (4) the length in days of the rise of antibody to peak titer, and the constants (5) for the first phase of antibody rise (k_1) and (6) for the derived k_1 when the rise consisted of more than one phase. Standard errors are also given for the last five of these measures in table 1, but not for the mean arithmetic peak titers because of the skewed frequency distribution of the latter (see W. H. and L. G. Taliaferro, 1954a and 1956). The mean arithmetic peak titers are used in the following analysis to show relative differences, but the mean log peak titers are used to estimate probabilities for differences between peak titers.

In figures 3, 4 and 5 semilogarithmic plots of daily mean arithmetic antibody titers for each group of the three experiments, except for groups 1-A5 and 1-B5, are shown. To obtain these graphs, mean daily arithmetic titers were obtained for a particular group from the fitted semilogarithmic curves of each rabbit of the group. The mean peak titers listed in table 1 are higher than shown in the average curves since the peak titers of the individual rabbits of a group rarely occurred on the same day. The differences between other means in the table and those shown by the graphs can be explained in a similar manner.

Experiment 1: Shielding or irradiation of the spleen or of the appendix of rabbits one day before the intravenous injection of heated red cell stromata.—Shielding the spleen (1-A1) or the appendix (1-B1) while irradiating the body with 500 r resulted in a marked protection of the hemolysin-producing mechanism. The totally irradiated control group (1-A3) reached a mean peak titer of only 110 hemolytic units/ml, while the spleen-shielded group attained 1400 units and the appendix-shielded animals the high value of 13,580 units. Both mean log peak titers were significantly higher than of group 1-A3 ($P = 0.01$ and 0.001, respectively). When comparing these peak titers to that of the nonirradiated group 1-A4 (7140 units), the interesting fact emerged that spleen-shielding resulted in a protection of only 18%, while appendix-shielding afforded complete protection with a normal mean peak titer. This conclusion was reached by comparing the mean arithmetic peak titers of groups 1-A1 and 1-A4 after subtracting from each mean that of group 1-A3. (The same procedure was employed in calculating the protection percentages to be quoted below.) The mean log peak titer of the spleen-shielded group was significantly lower than that of the nonirradiated controls ($P = 0.008$). Although the mean arithmetic peak titer of the appendix-shielded group (1-B1) was larger than the nonirradiated group, there was no significant difference in the mean log peak titer.

It is also noteworthy that the hemolysin rise occurred at a much higher rate in the appendix-shielded rabbits than in the spleen-shielded animals. Although the mean induction period was longer and the mean first-phase rate of antibody rise was less in both protected groups as compared to the normal rabbits (1-A4 and 1-B4), the reverse was true when they were matched with the totally irradiated groups (1-A3 and 1-B3). Thus, the mean induction period

of group 1-A4 (no X rays) was 2.6 ± 0.1 and of group 1-A3 (500 r to the total body) was 13.0 ± 1.1 days, whereas those of groups 1-A1 (spleen-shielded) and 1-B1 (appendix-shielded) were 4.6 ± 0.5 and 5.5 ± 0.2 days, respectively.

By irradiating the spleen or appendix with 1400 r (1-A2 and 1-B2), we attempted to simulate the effects of splenectomy or appendectomy (1-A5 and 1-B5). As compared to group 1-A4 (no X rays), the spleen-irradiated animals showed an unexpected elevation of the mean peak titer (7140 units for group 1-A4 and 12,800 units for group 1-A2). The differences between the log peak titers, however, were not significant ($P = 0.07$). The spleen-irradiated group showed, in addition, a significantly shorter mean antibody rise ($P = 0.04$) and a significantly longer induction period ($P = 0.001$). This lengthening of the induction period was the only feature which the spleen-irradiated and splenectomized animals had in common.

X-raying the appendix, on the other hand, did not have any significant effect on the immune response. Appendectomy, however, caused some depression in hemolysin formation as evidenced by a lowering of the mean log peak titer which approached significance ($P = 0.06$), an increase of the length and a decrease of the rate of the antibody rise to peak titer as compared to group 1-A4.

The results of experiment 1 are summarized in figure 3. The average curves illustrate the partial protective effect of spleen-shielding and the normal mean peak titer attained by the appendix-shielded group.

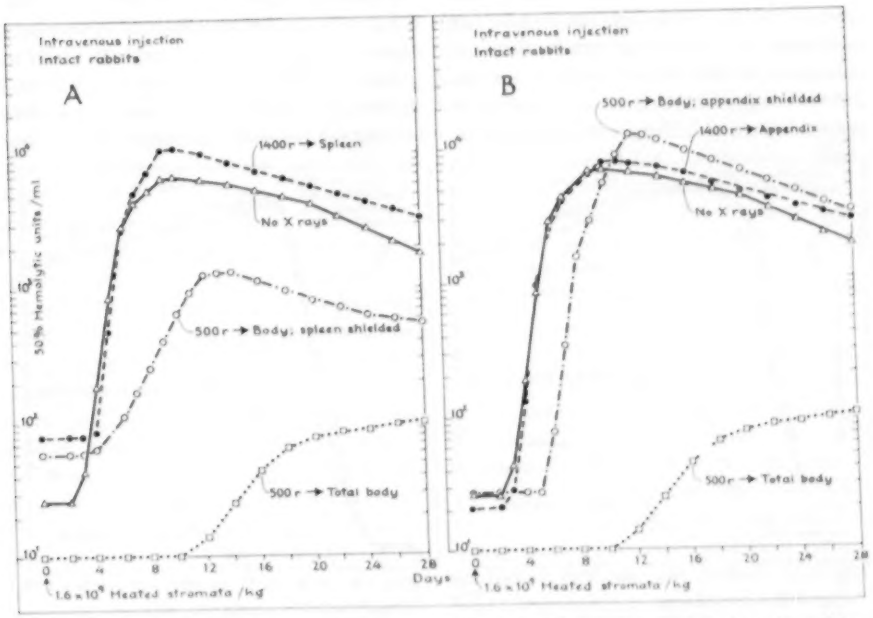

FIG. 3.—Mean hemolysin curves showing the effect of shielding or irradiating the spleen (A) or the appendix (B) as compared to totally irradiated and nonirradiated rabbits (exp. 1).

Note the partial protection of the hemolysin response due to spleen-shielding and the complete protection to peak titer by appendix-shielding as compared to the nonirradiated rabbits.

Experiment 2: Shielding or irradiation of the liver of intact or appendectomized-splenectomized rabbits one day before the the intravenous injection of heated red cell stromata.—Shielding the liver during 500 r body-irradiation resulted in a marked protection from the effects of X rays on the immune response, especially in the intact animals (2-A1). Thus group 2-A1 showed a significant elevation of the mean log peak titer over that of the totally irradiated controls ($P = 0.02$) and a mean arithmetic peak titer of 1200 units as compared to 110 units for the intact, totally irradiated group (2-A3). The mean arithmetic peak titer of the liver-shielded animals represented 16% of that of the nonirradiated controls (2-A4). This percentage is of the same order of magnitude as that attained by the spleen-shielded rabbits.

Removal of the appendix and spleen (group 2-B4) produced no significant reduction in mean log peak titer as compared to the intact controls (group 2-A4). When these organs were removed prior to liver-shielding with the rest of the body irradiated with 500 r (group 2-B1), the antibody titers were lower than in the intact, liver-shielded animals (group 2-A1) but there was no significant difference in mean log peak titer.

The mean induction periods were significantly shortened in both liver-shielded groups as compared to those of the respective totally irradiated animals. The mean length of the antibody rise to peak titer was not significantly different from normal in either of the liver-shielded groups.

The irradiation of 75% of the liver of intact animals (2-A2) caused a reduction in the length of antibody rise to peak titer ($P = 0.01$). This observation correlates with the already mentioned results obtained by extirpating the same part of the liver (Süssdorf and Draper, unpublished results). No changes from the normal response were observed after the irradiation of the liver of appendectomized-splenectomized rabbits (2-B2).

Figure 4 illustrates the effects of liver-shielding and liver-irradiation on the hemolysin response as described in this

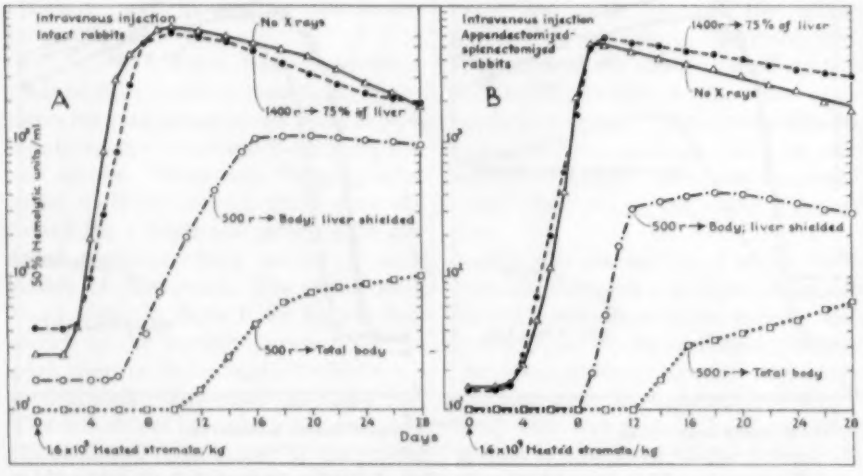

FIG. 4.—Mean hemolysin curves of the liver-shielded, liver-irradiated, totally irradiated and non-irradiated groups of experiment 2 in intact (A) and appendectomized-splenectomized rabbits (B).

Note the partial protection by liver-shielding especially in the intact animals and the essentially normal responses of the liver-irradiated groups.

section. The partial protection by liver-shielding in both intact and appendectomized-splenectomized rabbits and the essentially normal responses of the liver-irradiated groups are shown.

Experiment 3: Shielding or irradiation of the hind legs of intact or splenectomized rabbits one day before the intravenous or subcutaneous injection into the two hind foot pads of heated red cell stromata.—Intravenous injection following shielding of the hind legs during 500 r X radiation of the rest of the body resulted in significant protection in both the intact and splenectomized animals (3-A1 and 3-B1). Leg-shielding after splenectomy afforded complete protection to the amount of antibody formed when compared to group 3-B4 (splenectomized, nonirradiated rabbits) and partial protection when performed on intact animals as compared to group 3-A4 (intact, nonirradiated rabbits). In the latter case, the mean peak titer of group 3-A1 (510 units) was only 18% of that of group 3-A4 (2490 units). This percentage is comparable to those obtained by liver-shielding intact animals in experiment 2 (16%) and by spleen-shielding in experiment 1 (18%). Shielding the legs after splenectomy (3-B1) resulted in an arithmetic peak titer five times as high as that of group 3-B4, but there was no significant difference in mean log peak titer. It should also be noted that although the mean arithmetic peak titers of the two leg-shielded groups were widely different, there was no significant difference between the mean log peak titers of the two groups.

Irradiation of the hind legs of intact, intravenously injected animals with 1400 r (3-A2) did not alter the immune response greatly as compared to that of the nonirradiated controls (3-A4). The only significant difference between these groups was a delay in reaching peak titer by the leg-irradiated animals due to a lengthening of the mean length of the antibody rise ($P = 0.02$). Removal of the spleen before the irradiation of the hind limbs (3-B2) resulted in a significant rise in the log peak titer ($P = 0.04$) as compared to that of the splenectomized, nonirradiated group (3-B4).

Injecting the antigen into the hind foot pads of intact, leg-shielded animals (3-C1) did not produce a higher mean peak titer as compared to that of the totally irradiated controls (3-C3), although the induction period was shortened. With respect to the nonirradiated animals (3-C4), the response of group 3-C1 was depressed and delayed. The splenectomized leg-shielded rabbits (3-D1) showed a similar relationship to their respective totally irradiated and nonirradiated controls (3-D3 and 3-D4).

The irradiation of the hind legs followed by an injection of antigen into the hind foot pads caused some retardation of the hemolysin response in both the intact and splenectomized animals (3-C2 and 3-D2) as compared to the corresponding nonirradiated controls (3-C4 and 3-D4). This delay was evidenced by a decreased mean rate of the derived antibody rise in both leg-irradiated groups ($P = 0.001$) and a lengthened mean induction period in group 3-C2 ($P = 0.002$).

Finally, the evaluation of the results of experiment 3 revealed the interesting fact that the depression of the hemolysin titer caused by splenectomy followed by an intravenous injection of antigen was significantly greater than that observed following injection into the hind foot pads. Thus, the mean log peak titer in splenectomized animals receiving intravenous injections of antigen (group 3-B4) was significantly lower ($P = 0.01$) than that in splenectomized animals receiving injections of antigen into the hind foot pads (3-D4).

Figure 5 summarizes the results of

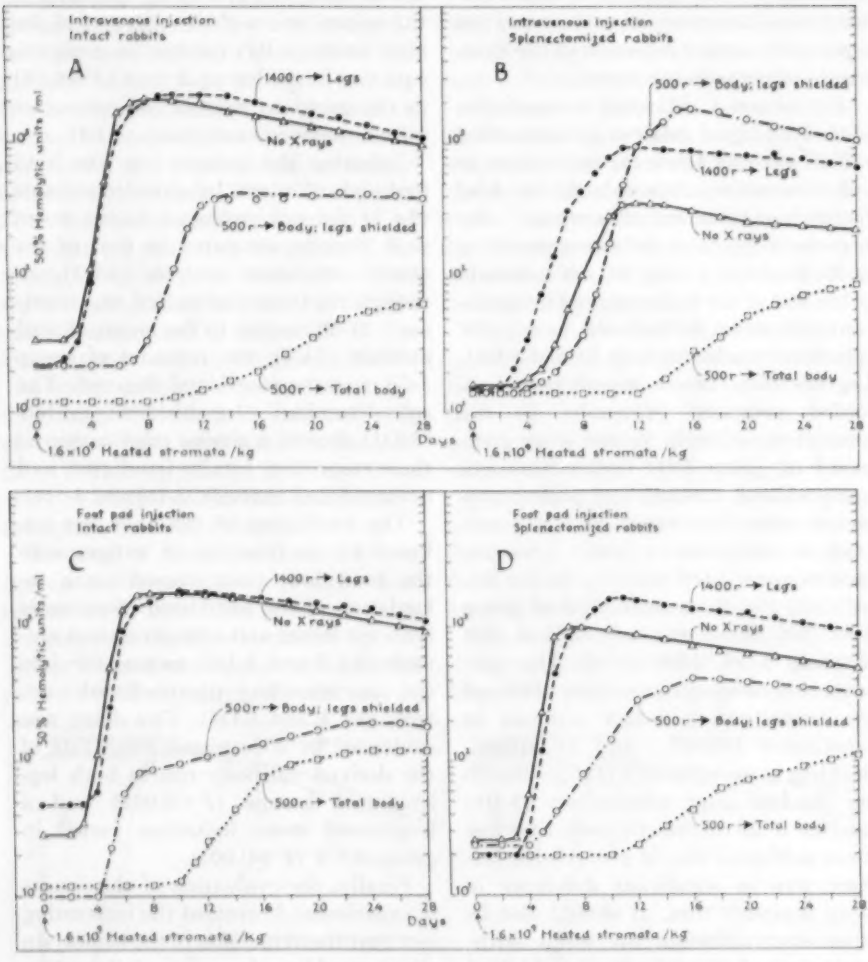

FIG. 5.—Mean hemolysin curves of the leg-shielded, leg-irradiated, totally irradiated and non-irradiated groups of experiment 3. Intact rabbits (A) and splenectomized rabbits (B) were intravenously injected. Intact (C) and splenectomized (D) rabbits which were injected into the hind foot pads are also shown.

In the intravenously injected rabbits, leg-irradiation after splenectomy had a stimulatory effect as compared to the splenectomized, nonirradiated controls. The depressive and delaying effect of splenectomy on the antibody response of nonirradiated rabbits were less if the antigen was injected into the hind foot pads (compare the nonirradiated groups in B and D).

experiment 3. It can be seen that leg-shielding had a greater protective effect in the intravenously injected animals (fig. 5A and B) than in the subcutaneously injected rabbits (fig 5C and D). Leg irradiation of splenectomized, intravenously injected animals stimulated the antibody response with respect to that of the splenectomized nonirradiated group (fig 5B).

DISCUSSION

The present results should be discussed in relation to (1) the efficacy of lead-shielding the spleen, appendix, liver and hind legs, (2) the effect of splenectomy, appendectomy and combined appendectomy and splenectomy on the formation of antibody in nonirradiated and irradiated animals and (3) the effect of 1400 r X irradiation to these organs or sites. The results are directly comparable to those by W. H. and L. G. Taliaferro (1956) in that in both studies, anti-sheep hemolysins were titrated by photometric methods using the 50% end point. Although we employed heated stromata whereas the Taliaferros employed whole red cells, both studies probably involved only the Forssman hemolysins (Talmage et al, 1956). We used about 8 times as much antigen as they did (1.6×10^9 stromata per kg rabbit). Our irradiated animals were injected 1 day instead of 2 days after irradiation. This time of antigen injection was employed because it is the approximate low point in the ability of the rabbit to form antibody after X radiation. If the effects of the timing of X radiation and of the antigen dose are additive, the work of the Taliaferros (1954 a and b) indicates that our log peak titers would have been about 0.55 log higher if our animals had been injected on the second day after X rays.

In group 1-A1, which is directly comparable to one of the series of W. H. and L. G. Taliaferro (1956), the results are in reasonable agreement. They found that a partial protection (52%) of the immune response was afforded by lead-shielding the surgically exteriorized spleen in contrast to the more complete protection reported by Jacobson et al (1950). The immune response of the spleen-shielded group in the present investigation showed a mean peak titer significantly higher than that of the totally irradiated control group but markedly lower than that of the nonirradiated group. The percentage protection calculated after adjusting the mean arithmetic peak titers of the normal and spleen-shielded groups by subtracting from each the mean arithmetic peak titer attained by the totally irradiated controls was 18%.

The protection expressed as percent peak titer, calculated as described in the preceding paragraph, varied among the intact, intravenously injected groups as follows: appendix-shielded, 100%, spleen-shielded, 18%, hind legs-shielded, 18%, and liver-shielded, 16%. It would appear, therefore, that lead-shielding of such diverse organs or sites as the spleen, liver and hind legs gives practically the same partial protection, whereas the appendix gives complete protection.

Lead-shielding of the various organs or sites of intact animals also resulted in a shortening of the induction period as compared to the totally irradiated controls. The length of the antibody rise to peak titer and the rate of its formation were significantly shortened and increased, respectively, except in the liver-shielded rabbits in which there was no significant difference from the totally irradiated rabbits.

Splenectomy, before intravenous injection of antigen, resulted in a significant lowering of the mean log peak titer and a reduction of the mean arithmetic

peak titer to approximately 6% of the intact controls. In marked contrast, splenectomy, before injection of the antigen into the hind foot pads, resulted in a lowering of the mean arithmetic peak titer to about 50% of the corresponding intact control group. This is similar to the results reported by Rowley (1950) and Wissler et al (1953) for the rat in which they found that splenectomy had little, if any, effect on the peak titer when antigen was administered intraperitoneally as compared to the depressed peak titer observed following splenectomy with intravenous injection of antigen. Appendectomy and combined appendectomy and splenectomy did not result in any significant lowering of the mean log peak titer as compared to the intact and splenectomized controls, respectively. In the case of appendectomy, the reduction in mean log peak titer approached significance ($P = 0.06$) and the mean arithmetic titer was reduced to 60% of the intact group. If further work proves this to be significant, it would be contrary to the findings of W. H. and L. G. Taliaferro (1950 and 1951).

The combination of splenectomy (3-B3) or of splenectomy and appendectomy (2-B3) with total body X irradiation did not significantly lower the mean log peak titer as compared to the intact, total body irradiated controls. This indicates that after total body irradiation (500 r) the spleen does not retain any appreciable capacity to form antibody. This conclusion is at variance with that of W. H. and L. G. Taliaferro who found an additive effect of splenectomy and total body irradiation.

There is no correlation between the degree of protection afforded by a shielded tissue and its ability to form antibody as indicated by the results obtained by extirpating the spleen or appendix. Marked reduction in the normal antibody response was observed when antigen was administered in a single intravenous dose after splenectomy (1-A5) and yet shielding the spleen gave only 18% protection. Appendectomy, on the other hand, was followed by a normal induction period and a hemolysin response with comparatively little depression and only a small delay of the response. Shielding the appendix, nevertheless, gave 100% protection. The results indicate that lead-shielding affords protection indirectly and does not necessarily directly protect the tissues involved in the actual formation of antibody under the conditions of these experiments. This indirect protection of antibody-forming tissues is most probably due to the shielding of mobile cells which can repopulate the antibody-forming sites and/or some acellular material such as the restoration principle described by Jaroslow and Taliaferro (1956) in yeast autolysate and HeLa cells.

Irradiation of the spleen, liver, appendix or hind legs with 1400 r while shielding the rest of the body in no case depressed antibody formation. In all cases except the liver, the irradiated animals formed more antibody but the increases were not statistically valid. This suggests that higher doses of X rays to the various sites might have stimulated antibody production as described by W. H. and L. G. Taliaferro (1956) for the spleen.

In the case of the liver, administration of 1400 r to 75% of the livers of intact rabbits revealed a significantly shortened period of antibody rise to peak titer. This result and the similar observation following partial hepatectomy (Süssdorf and Draper, unpublished results) suggest that irradiation (or partial removal) of the liver reduces the ability of the organ to metabolize a

portion of the antibody globulin as it is formed and results in a more rapid attainment of peak titer. Also, partial irradiation or extirpation may limit the capacity of the liver to phagocytose the particulate antigen and thus allow greater concentration of the antigen in more efficient antibody-forming sites. It is evident, then, that the liver plays only a slight, if any, role in the formation of antibody although it was observed (Süssdorf and Draper, unpublished results) that the liver may become more important after repeated antigen injections. Thorbecke and Keuning (1956) have reported recently that rabbit liver samples have the ability to form small amounts of antibody in vitro after repeated antigen injection of the donor rabbit.

SUMMARY

The spleen, appendix, liver or hind legs of rabbits were shielded from X rays or were X-radiated 1 day before one injection of 1.6×10^9 heated sheep erythrocyte stromata per kg. All injections are intravenous except for leg-shielded or leg-irradiated animals and their corresponding controls which received either intravenous or hind foot pad injections. Intact, splenectomized and appendectomized-splenectomized rabbits were used. Serum hemolysin levels were determined photometrically by employing the 50% end point titration method.

1. Shielding of the various organs or sites during 500 r body-irradiation resulted in the following protection expressed as percent peak titer: appendix-shielding, 100%, spleen-shielding, 18%, leg-shielding, 18% and liver-shielding, 16%. The marked depression of antibody formation after splenectomy and the relatively slight effect of appendectomy indicate that the protection of antibody-forming tissues is indirect. The protection appears to be due to the shielding of mobile cells which can repopulate the antibody-forming sites and/or some acellular principle present in the shielded areas.

2. Irradiation of the spleen, appendix, liver or hind legs with 1400 r X rays while shielding the rest of the body in no case depressed antibody formation.

3. Significant depression of peak hemolysin titer was observed after splenectomy in nonirradiated rabbits injected into the hind foot pads but not as markedly as in those injected intravenously.

4. Splenectomy, when combined with total 500 r body irradiation, did not have an additive effect in suppressing peak titer.

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EXPERIMENTAL TRANSMISSION OF *PASTEURELLA TULARENSIS* BY THE TICK, *DERMACENTOR PARUMAPERTUS*

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Since the discovery of natural infection of *Pasteurella tularensis* in the tick *Dermacentor andersoni* by Parker, Spencer and Francis (1924), other species have been involved as actual or potential vectors of tularemia. Parker (1933) and his associates demonstrated survival in and transmission by the adult rabbit tick *Dermacentor parumapertus* Neumann. Philip and Hughes (1953) reported naturally infected ticks of this species from the southwestern United States, and Woodbury and Parker (1954) reported naturally infected ticks of the same species from the Great Salt Lake Desert area of Utah. From these data, it seems likely that *D. parumapertus* plays an important part in the maintenance of tularemia in nature in the Great Basin region. The purpose of the following experiments was to determine if larval and nymphal *D. parumapertus* are capable of transmitting this disease under experimental conditions.

MATERIALS AND METHODS

Past. tularensis (Schu strain), stored in casein hydrolysate suspension at 0 to 4 C, was used in these experiments. The lethal virulence (LD_{50}) value was one organism as determined by the method of Goldberg et al (1954) from 10-day mortality data in mature guinea pigs, white-footed deer mice, *Peromyscus maniculatus*, and kangaroo rats, *Dipodomys microps* and *D. ordii*, following subcutaneous injection of graded doses of virulent cells. The bacteria were cultured on glucose-cysteine-blood agar at 37 C for 48 to 72 hours. Dilutions for inoculations were prepared in 0.5% gelatine in 0.85% saline.

The larval and nymphal ticks that were used in these experiments were reared from eggs deposited by engorged females taken from black-tailed jack rabbits shot in the field. Samples of these immature ticks were shown to be free from tularemia infection by animal inoculation. Both uninfected and infected larval and nymphal ticks were stored in desiccator jars at a relative humidity of 81% at room temperature when they were not on the host. Adult kangaroo rats were used as the experimental host animals.

The ticks became infected by feeding on kangaroo rats inoculated subcutaneously with approximately 2000 cells of *Past. tularensis* organisms. Fifteen lots, each containing from 500 to 1000 ticks, were placed on the rats the same days that the rats were inoculated; four lots, each containing from 500 to 1000 ticks were applied 2 days prior to inoculation. Upon the death of the host animals, heart, liver, and spleen tissues were cultured on plated agar medium. Organisms from the plates were identified as *Past. tularensis* by the slide agglutination method or by inoculation into white-footed deer mice.

After feeding on infected animals, ticks were checked for the presence of tularemia organisms. For the purpose of external disinfection, ticks were washed for 2 minutes in one part of Roccal (a quaternary ammonium compound) per 5000 parts of water and rinsed several times in sterile water. Five to ten ticks from each lot were ground with normal saline in Ten Brock tissue grinders and homogenates from each pool were inoculated into three white-footed deer mice. Tularemia infections in the mice were diagnosed by culture of the bacteria from infected groups.

Engorged ticks recovered from infected rats were held for 9 to 19 days after molting to the succeeding stage. Prior to application, ticks of some lots were washed in Roccal, whereas others were not. At this time they were applied to uninfected kangaroo rats. The same methods of diagnosis of tularemia infections were employed for tick-infected animals as previously discussed.

RESULTS

The data show that many of the ticks of each lot became infective (tables 1

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TABLE 1.—*Experimental infection of the tick,* D. parumapertus, *during engorgement on tularemia-infected kangaroo rats.*

Host		Tick		
Days from injection of *Past. tularensis* to death	Diagnosis for tularemia	Number of repetitions	Stage	Diagnosis for tularemia
3	+	1	Larval	–
3	+	9	Larval	+
4	+	6	Larval	+
4	+	2	Nymphal	+
5	+	1	Nymphal	+
3	+	1	Nymphal	+

and 2). In only one case did all the ticks of one series fail to become infective by feeding on an infected kangaroo rat. In this one instance, blood samples taken from the kangaroo rat showed that the larval ticks were removed before bacteremia developed in the host. No cultures of *Past. tularensis* were obtained from macerated control ticks. Ticks that became infective in the larval and nymphal stages retained *Past. tularensis* through the succeeding molts to nymphs and adults, respectively. Nymphs transmitted infection to kangaroo rats after 98 days, and it is suspected that this period may extend to 121 days (table 2). Adults were found to retain the organisms as long as 30 days, but in the case of 59 specimens, there was no evidence that they retained the infection or transmitted it to two cottontail rabbits, *Sylvilagus nuttallii*, 71 to 88 days after becoming infective. No difference was observed between externally disinfected and nondisinfected ticks in their ability to transmit infection. A single tick was capable of transmitting fatal infection. There were six instances when ticks taken from a presumably infected series failed to transmit the infection to kangaroo rats.

TABLE 2.—D. parumapertus *transmission of* Past. tularensis *to Kangaroo rats.*

Days between infection‡ of ticks and application to uninfected rats	Number of host animals	Ticks applied per animal (Nymphal stage)	Days from application of ticks to death of host	*P. tularensis* isolated from hosts
26	4	1	11–13	+
	3	7–10	11–14	+
	3	15	6–12	+
	1	15	10	*
	1	16	5	*
	2	20–31	11	+
	3	1	8–15	+
	4	5	7–15	+
	1	29	6	+
98	1	15	16	*
	2	39–42	12	+
121	1	10	13	*

* No proof of transmission; diagnosis not possible because of tissue deterioration.

† Date of tick infection is designated here as the date of death of infected host on which ticks fed.

DISCUSSION

The results of these experiments clearly show that larval and nymphal ticks of the species *D. parumpertus* were efficient vectors of *Past. tularensis* under the experimental conditions described. Previous work has shown these ticks to be naturally infected. These data demonstrate the importance of this ectoparasite in maintaining tularemia in nature.

Larvae and nymphs of *D. parumapertus* require 4 and 6 days, respectively, to engorge. The longevity of an infected kangaroo rat is approximately 4 days. It is likely that the ticks transmit the disease within 2 days after they attach. The extended longevity periods of the rats as shown in table 2 may have been due to a delay in attachment of infective ticks. There was little correlation between the number of ticks placed on an animal and the longevity of the infected host. As was expected, a single tick was as effective as 30 or more in transmitting fatal infection. In the six cases where the kangaroo rats did not become infected from ticks, it is assumed that the ticks may not have attached or may not have been infective.

Factors such as a delay in attachment and incomplete engorgement pose a problem worthy of mention. Should an infected host on which a larval or nymphal tick is feeding die before the tick engorges sufficiently to develop to the

succeeding stage, the tick may seek another host. Should this tick become infected from the first host, it may transmit the disease to an uninfected animal. Bell (1945) found that infected *D. variabilis* feeding on uninfected hosts lose their infection, but not without inoculating the host which subsequently may develop septicemia and infect other ticks feeding on it.

The results obtained with the adult ticks on cottontail rabbits are not conclusive. No tests were made to determine whether the ticks actually retained *Past. tularensis* at the time of application to the rabbits or after removal.

Although stage-to-stage transmission was demonstrated in *D. andersoni* by Parker, Spencer and Francis (1924), and in *D. silvarum* by Golov (Zasukhin, 1936), as far as is known this is the first time that trans-stadial transmission of *Past. tularensis* has been shown in *D. parumapertus*. Parker and Spencer (1926) proved transovarial transmission of *D. andersoni;* Bell (1945) maintained that transovarial transmission in *D. variabilis* is the exception rather than the rule. Further research is needed to determine if this type of transmission occurs in *D. parumapertus*.

SUMMARY

It is known that ticks of the species *Dermacentor parumapertus* may be found naturally infected with *Pasteurella tularensis*, and the adults may transmit the infection. Noninfected larval and nymphal ticks reared in the laboratory became infected by feeding on infected kangaroo rats. These ticks retained *Past. tularensis* through the succeeding molt, and the nymphs transmitted the infection to noninfected kangaroo rats. A single tick was capable of transmitting the infection. Nymphs transmitted the infection to kangaroo rats after 98 days. Adults were found to retain the organisms for as long as 30 days, but did not transmit fatal infection to cottontail rabbits 71 to 88 days after becoming infective. These data demonstrate the potential importance of this tick in maintaining tularemia in nature.

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PERITONEAL LEUKOCYTES IN MICE FOLLOWING X RADIATION, INFECTION, AND/OR INDUCED STERILE INFLAMMATION

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INTRODUCTION

Total and differential white blood cell (WBC) counts of the peripheral blood were studied by several investigators to elucidate the role of leukocytes in endogenous and exogenous infections of irradiated animals.[1] While some reported an excellent correlation between the peripheral granulocyte count of irradiated mice and the percent of animals surviving intravenous inoculation of *Pseudomonas aeruginosa*,[2] others did not find a consistent correlation between these counts and resistance to intranasally administered *Streptococcus zooepidemicus* or intraperitoneally administered *Salmonella enteritidis*.[3,4] Some of the latter investigators and others, independently, indicated that although granulocytopenia induced by radiation could be a factor in the increased susceptibility to infection of irradiated animals, one should also consider in this connection the induced alteration in the function of granulocytes, as well as impairment of other defense mechanisms.[5–9] It was postulated that increased susceptibility to infection resulting from irradiation was due to complex phenomena and will require much more study to be understood[3,10]

The following investigation was undertaken to evaluate more fully certain aspects of the role of leukocytes in increased susceptibility of mice to experimental infection following exposure to sublethal ionizing radiation.

MATERIAL AND METHODS

Experimental animals.—Two-to-three month old male and female NAMRU[11] strain mice weighing 15 to 20 g were used exclusively in all but one of the experiments, where mice of the Wistar and Tumblebrook strains were also included. All the animals were obtained from a commercial breeder and appeared to be free of any detectable infection. Ralston-Purina Laboratory Chow and water were provided ad libitum. A few mice were sacrificed by severing the cervical vertebrae. However, by far the greater number of animals were sacrificed by subcutaneous inoculation of 0.3 ml of 1:10 saline dilution of Nembutal into the fold of the skin back of the neck.

Irradiation.—A Keleket deep therapy X-ray unit was used for irradiation. The radiation factors were as follows: 200 Kilovolt peak, 20 ma, filter 0.25 mm. Cu and 1.0 mm. Al, half value layer 0.75 mm. Cu, distance from target to skin 75 cm, output approximately 28.5 r per minute. All X-ray doses were measured in air.

Sterile inflammation.—Sterile inflammation

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was induced by intraperitoneal administration of 0.2 ml of ground glass suspension prepared as follows. Broken pieces of Petri dishes were cleaned in aqua regia, washed in water, ground with mortar and pestle and then sifted through graded sieves. Material that passed through #200 (Precision Scientific) sieve with 0.074 mm openings was autoclaved and used in the experiments. This powder was suspended in physiological saline to a known turbidity, comparable with that of 2×10^9 *S. enteritidis* organisms, as measured by a Klett-Summerson colorimeter with #54 filter.

WBC counts.—Total and differential WBC counts were determined according to standard procedures. Differential counts were made on a basis of 100 cells unless the total WBC count was extremely low, as just after irradiation, when only 50 or, upon occasion, 25 cells were counted. Peritoneal WBC counts are represented as the number of cells in the exposed peritoneal cavity of a just-sacrificed mouse to which was added 0.5 ml of sterile saline for "washing" the contents. All WBC counts were made by one person to minimize variation due to technique.

Microbial agent.—*Salmonella enteritidis* 1891 (IX, XII . . .g,m) was obtained from Dr. F. L. Adler, The Public Health Research Institute of the City of New York, Inc. and was identified as culture 64 of the Agricultural Experimental Station of the University of Kentucky. In order to reduce variation, a stock of the agent was prepared on glass beads in individual ampules by drying under vacuum. The contents of a single ampule were added to 10 ml tryptose broth pH 7.5, which, after static incubation for 10 to 12 hours at 37 C, constituted the first passage. The second passage was prepared by seeding 50 ml of the broth with 1 ml of the first passage and incubating at 37 C for 18 hours. This second serial passage was used in the experiments after first testing for purity. A standard suspension of the organism in saline was prepared and determined turbidimetrically on the Klett-Summerson colorimeter.[7] Bacterial counts were made by means of a modified method of Miles and Misra[12] on rabbit-blood agar and/or beef heart agar plates which were incubated aerobically at 37 C for 18 to 24 hours.

RESULTS

Comparison of peripheral and peritoneal leukocyte counts in mice following irradiation, infection, and/or induced sterile inflammation.—The results of initial experiments revealed that the concentration of WBC in the peritoneal washings of normal NAMRU strain of mice was $13,500 \pm 1700$/mm.[3] The number of peritoneal WBC was found to be independent of the method of sacrificing the experimental animals, i.e., using Nembutal or severing the cervical vertebrae. Differential peritoneal WBC counts disclosed the presence of about 75% lymphocytes, 25% monocytes and almost complete absence of granulocytes.

During the course of subsequent experiments one group of 2-to-3-month-old NAMRU mice were exposed to a high sublethal dose of 300 r whole body X radiation. Within 2 to 3 hours after exposure these animals and nonirradiated controls were inoculated intraperitoneally with approximately 1.5×10^6 *S. enteritidis* in 0.2 ml saline. Another group of mice was treated similarly, but was first given intraperitoneal inoculation of ground glass in 0.2 ml saline 1 day before irradiation in order to produce sterile inflammation. Total peritoneal and peripheral counts were determined 1 day after irradiation. Symbols designating the various groups of mice used in this experiment follow.

- NOO, normal untreated mice
- NGO, normal mice receiving ground glass
- NGI, normal mice receiving ground glass and *S. enteritidis*
- XGO, irradiated mice receiving ground glass
- XGI, irradiated mice receiving ground glass and *S. enteritidis*
- XOO, irradiated-only mice
- XOI, irradiated mice receiving *S. enteritidis*
- NOI, normal mice receiving *S. enteritidis*

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Mean peripheral and peritoneal counts of the various groups are shown in figure 1. Except for two groups noted later, all counts are the mean of from 11 to 25 animals. The counts shown on the left in figure 1 are those of the normal (NOO) animals. Inoculation of ground glass intraperitoneally initiated an inflammatory response which is indicated (a) by a marked leukocytosis, the WBC increasing from a normal level of 13,500 up to 32,300/mm³ in the peritoneal washings, and (b) by a seeming rise in the number of WBC in the peripheral blood as shown in the center section of the figure (NGO mice).

Ground-glass-inoculated animals and normal uninoculated controls, which were irradiated and infected, provided seven groups of mice. Those receiving ground glass (NGO, NGI, XGO, XGI) are shown in the upper part of the right hand column of figure 1. The remaining 3 groups (XOO, XOI, NOI), shown in the lower part of the same column were not treated with ground glass. Of the former, the uninfected animals inoculated with ground glass (NGO, average 5 mice) continued to show peritoneal and peripheral counts similar to those observed on the first day, i.e., 28,600 $\pm$3,400 and 13,800 $\pm$ 1,500/mm³, respecitvely. The peritoneal counts of the infected animals receiving ground glass (NGI) showed a great increase in WBC, 43,900 $\pm$ 5,900/mm³, with a slight drop in peripheral blood, 9,300 $\pm$ 810/mm³. The irradiated-only (XOO) animals showed the expected marked peripheral and peritoneal leukopenia: 3,600 $\pm$ 260/mm³ and 8,000 $\pm$ 780/mm³. However, while the peripheral counts of the irradiated-infected (XOI) animals were also at low levels 1 day after this treatment (2,800 $\pm$ 250/mm³), just as they were for the irradiated-noninfected (XOO) animals, the peritoneal count (26,100 $\pm$3,700/mm³) was markedly and significantly higher than that of the irradiated-only (XOO) or even the normal (NOO) animals (8,000 $\pm$ 780 and 13,500 $\pm$1,700/mm³ respectively). This observation is substantiated by the low peripheral (2,900 $\pm$ 210/mm³) and high peritoneal (26,900 $\pm$ 4,400/mm³) counts in irradiated ground-glass-inoculated (XGO) animals and also in ground-glass-inoculated animals which were irradiated and infected (XGI, average of 5 mice, 4,600 $\pm$ 1,300/mm³ peripheral

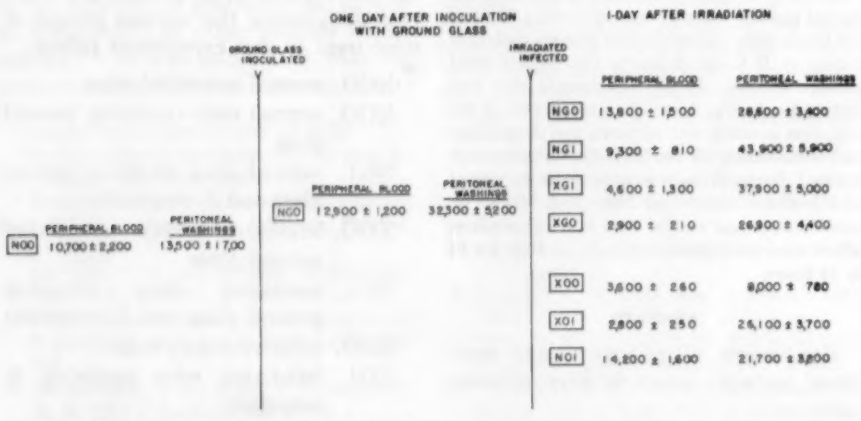
ONE DAY AFTER INOCULATION WITH GROUND GLASS
GROUND GLASS INOCULATED
IRRADIATED INFECTED
1-DAY AFTER IRRADIATION
PERIPHERAL BLOOD
PERITONEAL WASHINGS
NOO 10,700 ± 2,200 13,500 ± 1,700
NGO 12,900 ± 1,200 32,300 ± 5,200
NGO 13,800 ± 1,500 28,600 ± 3,400
NGI 9,300 ± 810 43,900 ± 5,900
XGI 4,600 ± 1,300 37,900 ± 5,000
XGO 2,900 ± 210 26,900 ± 4,400
XOO 3,600 ± 260 8,000 ± 780
XOI 2,800 ± 250 26,100 ± 3,700
NOI 14,200 ± 1,600 21,700 ± 3,800

FIG. 1.—Total peripheral and peritoneal WBC counts (mean and standard error of mean) in normal and irradiated mice.

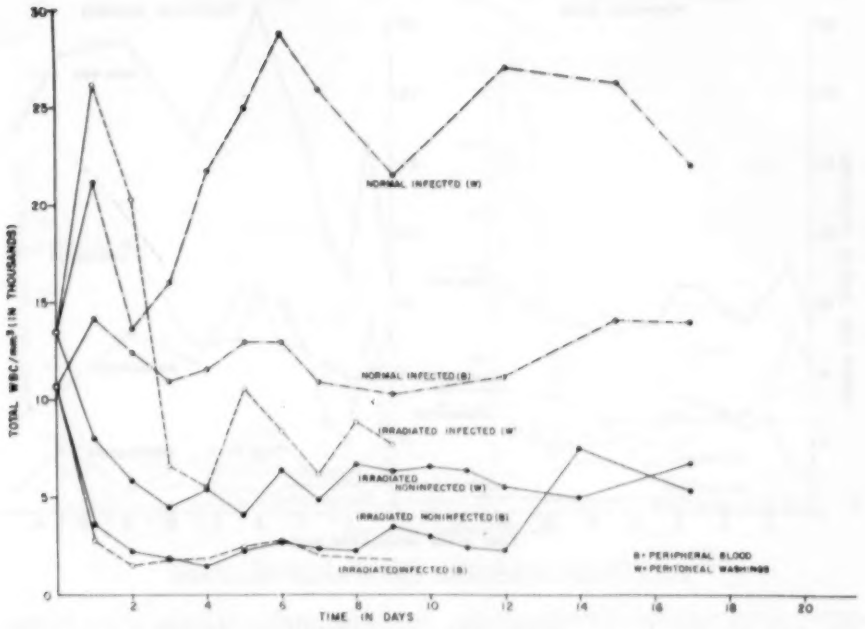

FIG. 2.—Total peripheral and peritoneal WBC counts in normal and irradiated mice.

and 37,900 ± 5,000/mm³ peritoneal). In contrast to this, the normal animals (NOI) also possessed a peritoneal leukocytosis, and showed a tendency towards leukocytosis in the peripheral blood as well.

Daily total and differential WBC counts in normal and irradiated mice challenged with S. enteritidis.—The peritoneal leukocytosis in irradiated animals 1 day after infection with *S. enteritidis* prompted daily observations of WBC changes (peripheral as well as peritoneal) in irradiated infected animals and their comparison with WBC counts of irradiated-only and normal infected mice.

Three groups of 9 to 10-week-old NAMRU mice were used in these experiments. Two of the groups were exposed to 300 r wholebody X radiation. One of the irradiated groups and the nonirradiated group were inoculated intraperitoneally with approximately 1.5×10^5 *S. enteritidis* in 0.2 ml of saline. The remaining group received no further treatment. Peripheral and peritoneal WBC counts were determined daily on six mice of each of the three groups. The results of total WBC counts in normal and irradiated mice are presented in figure 2.

The data show that peritoneal leukocytosis in the normal-infected animals (NOI) persisted during the 17 days of observation. This was not the case with peripheral WBC, although the total counts fluctuated in the upper part of the normal range and even made occasional excursions above it. The initial peripheral leukopenia in irradiated-uninfected (XOO) animals persisted for 12 days, after which time the counts began to show the expected signs of recovery. Peritoneal counts of these animals did not reach normal levels by

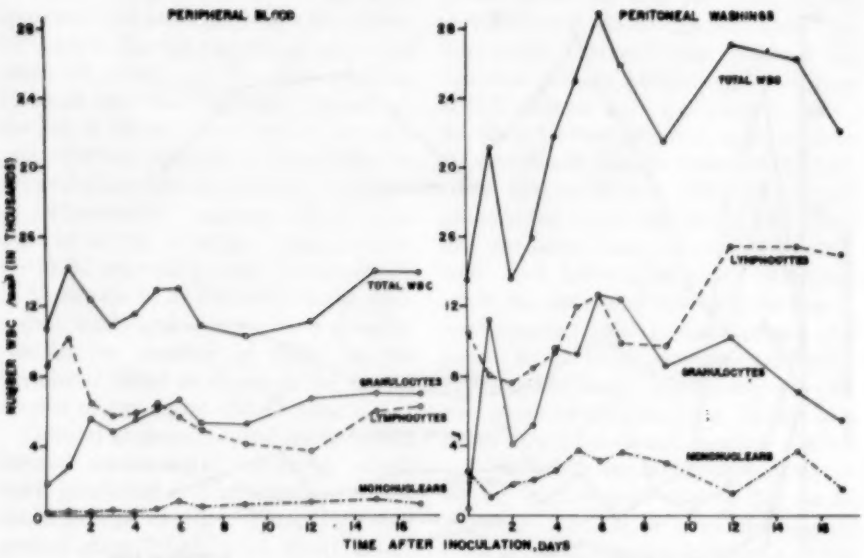

FIG. 3.—Total and differential WBC of normal infected mice.

the end of the 17-day observation period. A marked peritoneal leukocytosis of the irradiated-infected group (XOI) lasted only for 2 days, to decline precipitously on the 3rd day to a low level and hovering there for the remaining 7 days of observation.

Figures 3, 4, and 5 show the differ-

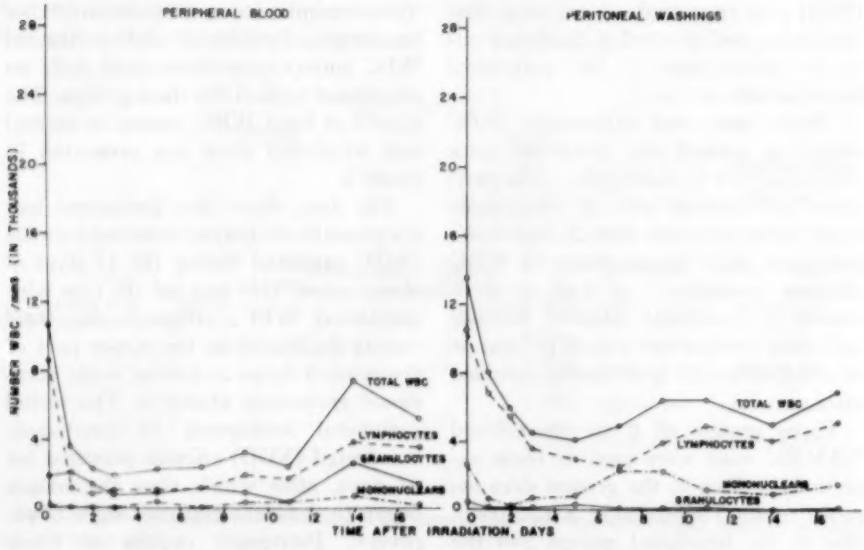

FIG. 4.—Total and differential WBC of irradiated noninfected mice.

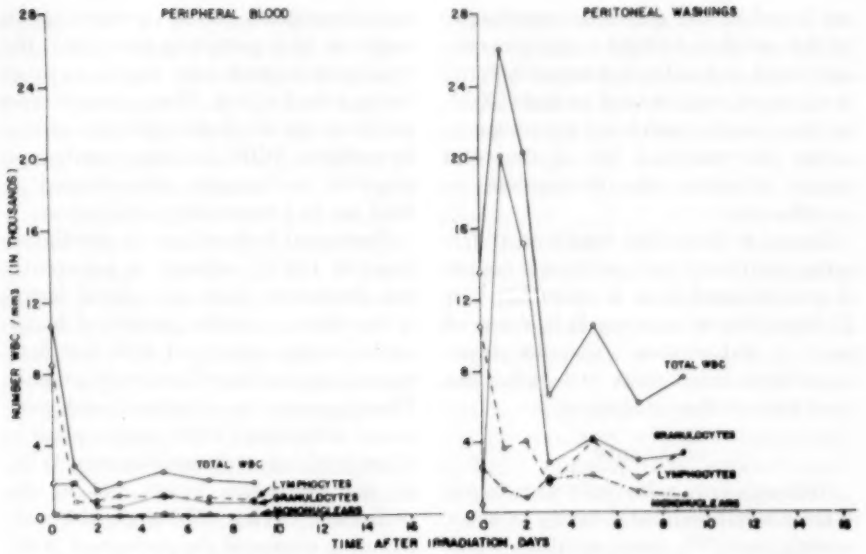

FIG. 5.—Total and differential WBC of irradiated infected mice.

ential counts of all the above groups of mice. In the normal-infected mice (NOI, fig. 3) both lymphocytes and granulocytes are responsible for the increase in leukocytes in the peripheral blood and peritoneal washings. The mononuclears do not show much fluctuation. In the irradiated-only mice (XOO, fig. 4) leukopenia in the peritoneal cavity is due almost entirely to the lymphopenia. (It should be noted that, normally, only very few granulocytes

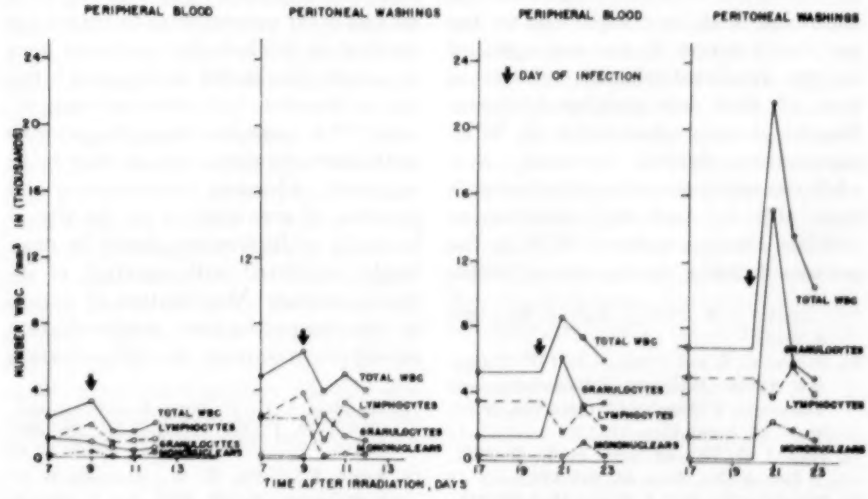

FIG. 6.—Total and differential WBC of mice infected 9 or 20 days after irradiation.

are found in the peritoneal washings.) In the peripheral blood a granulopenia also exists, as has been observed before.[3] In the irradiated-infected animals (XOI, fig. 5) a similar peritoneal lymphopenia occurs, the rise and fall of the total counts reflecting the fluctuations in granulocytes.

Figure 6 shows the total and differential peripheral and peritoneal counts of mice infected with *S. enteritidis* 9 or 20 days after irradiation. It is observed that a leukocytosis (granulocytosis) occurs after infection at 20 days but not after 9 days after irradiation.

DISCUSSION

Although only a few cells were found in the normal peritoneal cavity by some investigators,[13,14] concentrations comparable to those found by us have been reported.[15,16] In the present experiments, irradiated mice which had been previously inoculated with ground glass or were infected just after irradiation, or both, showed a marked peritoneal leukocytosis. This response was expected and was observed in the normal nonirradiated animals along with the maintenance of, or a slight rise in, the peripheral counts. It was not expected in the irradiated-infected animals in view of their low peripheral counts. Results of daily observation of WBC populations showed, however, that while the nonirradiated infected animals were able to, and did, continue to mobilize a large number of WBC in the peritoneal cavity during the entire 17-day observation period (presumably in response to a persisting infection), the irradiated animals were unable to do so beyond the 2nd day. These observations point up the irradiated animals' ability to mobilize WBC in large numbers in response to bacteria administered at least up to 3 hours after irradiation.

Peritoneal leukocytosis in irradiated-infected (XOI) animals is apparently not dependent upon peripheral leukocytes, since a similar peripheral leukopenia is also associated with low peritoneal counts of irradiated-only animals. Discrepancies in peripheral and peritoneal differential WBC counts point to absence of free exchange between WBC in the peripheral blood and in the peritoneal cavity. Although the contributing source of the peritoneal WBC is not elucidated by our observations, a likely explanation is offered by a recently enunciated concept " . . . that the majority of leukocytes which contribute to an acute leukocytosis are released from areas of storage."[17]

It has been suggested that the inflammatory response to intraperitoneal injection of powdered glass, talc, olive oil and other preparations increased the survival of midlethally irradiated mice by a reduction in the incidence of infection with certain indigenous microorganisms.[18] If it is assumed that phagocytosis contributes to resistance of mice to *S. enteritidis*, adequate concentration and function of granulocytes at the site of bacterial multiplication should be positively correlated with survival of infected animals. Mobilization of granulocytes during the early postirradiation period in response to inflammation

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should lead to a reduced microbial concentration, thereby rendering an infection potentially less dangerous to the host. This would not be the case in experimental infection initiated later, during the postirradiation periods lacking leukocytic response.

Our results indicate that in irradiated-infected (XOI) mice a granulocytosis in the pritoneal cavity reaches a peak within 24 hours after irradiation. Thereafter the decline in numbers of WBC is precipitous and granulopenia is observed from the 3rd postirradiation day on. Whether in spite of this condition a sufficient number of functional WBC is left to dispose of or prevent multiplication of even a small microbial inoculum (of the order of 10 to 100 organisms) is not known. Impaired bactericidal activity has been demonstrated in cell-free extracts of leukocytes from the irradiated animals.[6] Data on the relative function of "phagocytin,"[19] a recently described bactericidal preparation obtained from polymorphonuclear leukocyte and particularly active against certain gram negative enteric organisms, in normal and irradiated animals should shed additional light on mechanisms in pathogenesis of intraperitoneal infection initiated by *S. enteritidis* in these animals.

In irradiated-noninfected (XOO) mice daily observation of WBC revealed that a peritoneal and peripheral leukopenia occurred one day after irradiation and lasted through 17 days of observation. Impairment to the hematopoietic system was borne out by the leukopenia in mice infected with *S. enteritidis* 9 days after irradiation (fig. 6). The recovery from the temporary damage was evident when mice were infected 20 days after irradiation: the observed leukocytosis was associated with a decrease in susceptibility to *S. enteritidis* at this postirradiation period.[4] The postirradiation period and granulocyte counts have been shown to be associated with resistance of irradiated animals to experimental infection with *P. aeruginosa*.[2]

It has been indicated that while leukocytes are important in checking infection in local situations, their role in removal of bacteria from the blood stream "appears to be minimal."[20] In our experience, serial sacrifice of iradiated animals infected with *S. enteritidis* (in preparation) revealed bacteremia within 5 minutes after intraperitoneal inoculation indicating a transport of bacteria to reticulo-endothelial system (RES) clearing centers. The concentration of organisms in the peritoneal cavity dropped to a low level 11 hours after infection, and remained there for a period of 4 days. Thereafter, the number of bacteria in the peritoneal cavity increased markedly and was correlated with the fatality of infected animals until the 12th postirradiation day, after which time no organisms were recovered and no mice died. An initial peritoneal granulocytosis could account for the low peritoneal bacterial concentration during the early postirradiation period, while subsequent granulopenia could account for the multiplication of the organisms. However, since the effectiveness of removing bacteria from the blood by the RES altered by radiation[21] is probably of short duration[22] it may be that the organisms, after multiplication elsewhere, reinvade the blood stream

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and after being transported to the peritoneal cavity multiply therein unchecked, because of the lack of granulocytes.

These results show that under some conditions the total and differential leukocyte counts of the peripheral blood of an irradiated animal are not an index of the animal's leukocytic response to infection. Therefore, they should not be used exclusively or only warily so in correlative studies dealing with the susceptibility pattern of the irradiated animal.

SUMMARY

A study of the concentration and nature of WBC was made at the site of experimental infection in normal and in irradiated mice, in order to gain a better knowledge of the role of leukocytes in radiation injury and infection.

Large numbers of WBC were found in the peritoneal cavity of normal mice. About 75% of the cells were lymphocytes and 25% were monocytes; the number of polymorphonuclear leukocytes was negligible.

Intraperitoneal inoculation of *Salmonella enteritidis* or ground glass stimulated a slight increase in the concentration of peripheral leukocytes but caused a marked peritoneal leukocytosis. The peripheral and peritoneal leukocytosis occurred one day after infection and lasted for the duration of the experiment (17 days).

In contrast to the normal leukocyte response following infection, the peripheral counts of irradiated and subsequently infected animals showed a marked leukopenia resembling that of irradiated-only animals. But these peripheral counts of irradiated-infected mice did not reflect the pronounced peritoneal leukocytosis evident for 3 days after infection.

It is therefore suggested that in the early postirradiation period, correlation of peripheral WBC counts and the outcome of radiation injury may be misleading.

SEROLOGIC STUDIES OF ENCEPHALITIS IN JAPAN

I. HEMAGGLUTINATION-INHIBITING, COMPLEMENT-FIXING, AND NEUTRALIZING ANTIBODY FOLLOWING OVERT JAPANESE B ENCEPHALITIS

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The objective of the studies reported in this and the two following publications[1,2] was to study the formation and persistence of antivirus antibody after natural infection of humans by Japanese B encephalitis virus (JBE virus). This first paper reports antibody responses following overt encephalitis in American and Japanese patients. It supplements and extends previous studies of this subject[3–8] by the greater number of patients studied, the comparison of indigenous Japanese and American populations, and by the simultaneous use of four serologic techniques. It confirms the usefulness of the hemagglutination-inhibition (HAI) technique.[9–12] Later papers will report the antibody responses produced by clinically inapparent infections in Japanese school children, and evidence indicating the occurrence in Tokyo of encephalitis of other, as yet unknown, etiology concurrent with Japanese B encephalitis.

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The author is greatly indebted to many doctors and technicians without whose cooperation this study would not have been possible. The contributions of the following are particularly noteworthy: Dr. Keigo Uchiyama, Director of Komagome Hospital, Tokyo; Dr. Mannosuke Yokota, Chief of the Department of Medicine of Komagome Hospital; Dr. Masao Benoki, Chief of the Virus Diagnostic Section of the Otsuka Laboratory, Tokyo; and Lt. Leon Kutner of the 406th Medical General Laboratory.

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METHODS

Serial serum specimens from Japanese patients with encephalitis were obtained from the municipal hospitals of Tokyo. Serial serum specimens from healthy Japanese children were obtained from elementary schools of Iruma-gun, Saitama Prefecture, a rural district on the northwestern border of Tokyo-to. (Tokyo-to is a land area of about 800 square miles which includes much rural and suburban land in addition to metropolitan Tokyo.)

All serums were stored at −20 C until thawed for serologic studies. With few exceptions all serums from a single patient were tested simultaneously by each technique.

The HAI technique with Japanese B virus antigen was first developed by Sabin and Buescher[12] and further modified by Chanock and coworkers.[9–11] Briefly, it entails preparation by high speed centrifugation of a supernate from a suspension of brain from Japanese B virus-infected mice, incubation of 8 hemagglutinating units of this antigen with serial dilutions of serum from which interfering materials have been removed by acetone fractionation, and testing the ability of such serum-antigen mixtures to agglutinate erythrocytes from baby chicks. The presence of antibody is indicated by inhibition of the hemagglutinating capacity of the virus antigen.

The complement-fixation (CF) tests were performed by a standard technique[13] which utilized virus antigen prepared in mouse brain, two 100%-hemolysis units of complement, and overnight incubation at 4 C.

The virus dilution neutralization technique was the standard army technique[14] utilizing equal volumes of undiluted serum and serial 10-fold dilutions of virus. Virus dilution was carried out in a 10% serum-saline solution using undiluted unheated antibody-negative normal human serum to supply accessory substances.[11] This serum was stored at −20 C prior to use.

The serum dilution neutralization test was performed by preparing serial 10-fold dilutions of the serum to be tested, using undiluted normal serum as above for the diluent, and mixing each dilution with an equal volume of virus preparation. Two virus dilutions were so prepared as to give expected doses of 10 LD_{50} and 100 LD_{50} after incubation, as judged by prior titrations. The test was run in parallel using both virus dilutions, but calculations were based on the results with that virus dose which actually contained between 20 and 160 LD_{50}'s, as determined by simultaneous titration.

For both neutralization techniques the virus plus serum mixtures were incubated in a 37 C water bath for 2 hours. Mice were a non-inbred strain of white Swiss type obtained from commercial sources in Japan. They were about 30 days old and weighed 8 to 14 g (usually 8 to 10 g) when inoculated. Inoculum volume was 0.03 ml intracerebrally. Six mice were used per dilution. The Reed-Muench[15] formula was used for calculation of all 50% end points.

American and Japanese patients with a clinical diagnosis of encephalitis were divided into three categories on the basis of serologic studies: (1) established diagnoses—those with an established diagnosis of Japanese B encephalitis on the basis of a 4-fold rise in CF titer and/or a titer of 1:16 or greater (plus 1 case which was anticomplementary but showed a rise of over 1.0 log in neutralizing capacity to a maximum titer of 3.2 logs); (2) presumptive diagnoses—those with no CF antibody rise or with maximum CF titer less than 1:16, but with HAI antibody findings comparable to those seen in the above group; (3) no serological evidence of JBE virus infection—those patients hospitalized with a diagnosis of definite or suspected central nervous system infection, but with no evidence by CF, HAI, or neutralization techniques that the cause of disease was JBE virus. Only patients from whom serum was collected at least 21 days after onset were included in this last category. Patients in this category are excluded from the present discussion but will be considered separately in the third paper of this series.[2]

The group with a diagnosis of Japanese B encephalitis definitely established by CF antibody criteria included 23 American and 43 Japanese patients. Inspection of antibody data (table 1, fig. 1) showed that titers tended to be lower in Japanese patients than in Americans. In figure 1 all serums obtained 2 to 4 days after onset of illness were averaged and plotted at day 3; serums collected on days 5 to 9 were averaged and plotted at day 7; and days 10 to 19, 20 to 29, 30 to 39, 40 to 49, and 59 to 99 were averaged and plotted at days 14, 24, 34, 44, and 74, respectively. Serums plotted at day 150 were collected simultaneously in February 1955, between

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140 and 180 days after onsets. The number of serums from which average titers (geometric means) were computed varied considerably for the different techniques and different time periods because collection times were irregular and serum volumes were frequently too small to permit study by all techniques. The number of serums averaged for the first four time periods approaches the total number of patients as recorded for each technique in table 1 and is not less than 10 by any technique. The averages for later time periods represent as few as 2 serums, but the day 150 average for Japanese is based on 9 patients.

RESULTS

By HAI and CF techniques average titers were lower in Japanese patients than in Americans at all times. By the virus dilution neturalization technique the same tendency was apparent, but the differences were smaller and less consistent. Serum dilution technique gave identical results for Americans and Japanese. To test the significance of these differences standard deviations and critical ratios* were calculated using data from all serums collected later than the 9th day of illness. Earlier serums were excluded from these calculations in order to minimize differences due to error in timing onset of clinical disease and differences in *rate* of antibody formation; however when data from days 1 to 9 were included in the calculations the conclusions concerning statistical significance were unchanged. The differences in CF and HAI antibody levels between Japanese and American patients are highly significant (table 1), and the differences observed in the virus dilution neutralization titers is probably significant (critical ratio, 2.1). A similar discrepancy between HAI titers in American and Japanease patients is indicated in the 1953 data from this laboratory (reference 4, Table XI, p. 30).

The cause of the poorer antibody response of Japanese as compared to Americans is not apparent. The possibility of difference due to age was considered since Americans were older (average age 23.3 years, minimum 19 years) than Japanese (average 9.6 years), but this possibility seems to be excluded by the fact that among the Japanese patients HAI antibody response of patients over 20 years (5 patients) was not significantly different from the total Japanese group (critical ratio 0.3) and was significantly lower than the American group (critical ratio 4.9). Nutri-

* Advice and assistance in statistical calculations were generously given by Major Herbert Ley and Mrs. Mary Fish, Department of Epidemiology, 406th Medical General Laboratory. Critical ratio is a calculation of the probability of chance occurrence of the observed difference between two means (the difference between two means divided by the standard deviation of this difference). A value of 2.0 or 3.0 indicates that the observed difference might be expected by chance once in 20 times or 100 times respectively.

TABLE 1.—*Comparison of antibody reactions in Japanese and American patients with serologically confirmed Japanese B encephalitis. Computations based on all serums collected on day 10 through day 100.*

Technique	Japanese cases				American cases				Critical ratio
	Number of cases	Number of serums	Titers		Number of cases	Number of serums	Titers		
			Mean*	Range			Mean*	Range	
HAI	43	77	640	40–1280	23	37	1920	80–1500	5.9
CF	43	65	17	0–64	22	35	39	4–256	2.9
Virus dil. neut.	24	34	2.8	2.0–3.5	21	33	3.2	1.5–4.0	2.1
Serum dil. neut.	26	45	500	10–10,000	20	33	500	10–3200	0.0

* Titers are expressed as reciprocals of serum dilutions or as numbers of logs of virus. "Mean titers" were computed by assigning arbitrary weights in arithmetic progression to the antibody titers (e.g. for CF titers: <1:2 was 0, 1:2 was 1, 1:4 was 2, 1:8 was 3, etc.). The mean and the standard deviation were then computed from these figures using classical methods for data in arithmetic scales. The mean titers tabulated above were obtained by reconverting from the arbitrary scale to the corresponding serum or virus titer; thus these values are essentially geometric means.

tional or genetic factors are possible explanations since racial differences in other diseases are known to exist (e.g., breast cancer in the female is 7 times as frequent in Americans as in Japanese of equal age groups). Another possible explanation is that a more highly antigenic strain of virus caused the infection in Americans (a majority were from Okinawa, whereas all Japanese were from Tokyo).

HAI antibody became detectable soon after onset of illness, but early titers differed markedly. Several Japanese and American patients had titers of 1:320 and 1:640 within 5 days after appearance of symptoms, while a few of the Japanese patients remained negative (less than 1:40) as late as day 8. The discrepancy between Japanese and Americans was greatest by this technique (fig. 1 and table 1). In Americans the average titer reached 1:320 by day 3. By day 10 the lowest titer was 1:320, and the average was over 1:1280. Maximum titers over 1:2560 were rare. Japanese showed the same pattern but titers were lower. Titers of 1:160 were common as late as day 30, and only one-half of all serums collected after day 9 titered 1:320 or greater. Although average HAI titers did not rise significantly after day 10, a few patients did not reach maximum titers until the 3rd week. Some patients showed 2- to 4-fold drops in titer after 30 days, and all titers had decreased by 5 to 6 months after illness.

CF antibody was occasionally detectable at 1:4 by day 4, and occasionally reached 1:16 by day 7. However, a majority of both Japanese and American patients (63 percent of specimens) were

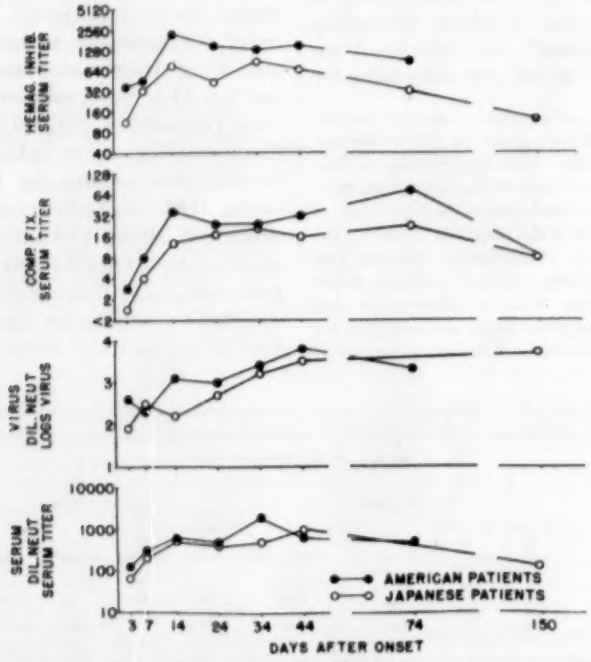

FIG. 1.—Comparison of average antibody response in Japanese and Americans following Japanese B encephalitis.

negative (less than 1:4) through day 10, and 3 Japanese were still negative between 15 and 50 days after onset. The mean CF titer was at or near maximum by 15 days after onset and showed only minor fluctuation during the next 2 months, but dropped significantly by 5 to 6 months after illness. These observations on time and magnitude of CF antibody rise are in general agreement with those of previous workers.[3-7]

Neutralizing antibody was detectable very early by the virus dilutions technique. Serums from only 2 patients (1 Japanese and 1 American) neutralized less than 1.5 logs of virus in the earliest serum specimen available (days 4 and 6). One patient neutralized 3.9 logs of virus 24 hours after first recognized symptoms. The average antibody titer in serums collected during the first 4 days after onset was 1.9 logs, and for serums collected on days 5 to 9 it was 2.5 logs. Thereafter average titers rose progressively but only slightly for the next 5 weeks to a maximum around 3.0 logs. There was no drop in titer by 6 months.

The serum dilution neutralization technique also showed high titers in early serums and progressive but slight rise in average titers in the next month or two. The drop in average titer by day 150 cannot be accepted without reservation since the neutralization tests on these late serums were not done simultaneously with the earlier specimens, and differences in the virus dose do materially affect the serum titers.

Comparison of the virus dilution and serum dilution neutralization data was possible on 30 patients from whom sufficient serum was obtained for study of serial specimens by both techniques. If a 10-fold rise in serum dilution titer is set as the ciriterion for diagnosis of current infection this technique established the diagnosis in 7 patients, of whom only 4 were diagnosable by virus dilution neutralization tests; but it failed to establish the diagnosis in another 3 who were diagnosed by virus dilution criteria (2.0 logs rise). If a 4-fold rise in serum dilution titer is accepted as significant (in tests performed simultaneously with identical virus inoculum), this technique established diagnosis in 12 patients of whom only 7 were diagnosable by virus dilution criteria, but again failed to establish the diagnosis in 3 patients who were diagnosed by the virus dilution neutralization test.

Both neutralization techniques failed to show a diagnostic rise in titer in 50% of patients who were diagnosed by both CF and HAI techniques.

A graphic comparison of averaged antibody curves as detected by these 4 techniques is presented for the first 3 weeks after onset in fig. 2.

In determining the value of a serological test for the diagnosis of current disease, the persistence of antibody is of as great importance as the time of its appearance, since antibody persisting from prior infection must be distinguished from antibody formed in response to the infection under study. Serums from 2 patients were obtained 12 to 15 months after Japanese B encephalitis as well as during illness and convalescence (table 2). Patient G.A. was infected in Tokyo, and S.V. in the Philippine Islands. Both were Caucasian. CF antibody titers were 1:4 or less. Neutralizing antibody titers remained high. HAI antibody had dropped markedly from previous levels, but the persistence of a titer of 1:320 at 15 months necessitates that a titer of 1:640 or greater be set as the minimum titer for presumptive diagnosis of current infection in the absence of a demonstrable rise in titer. Neutralizing antibody decreased slightly in one patient

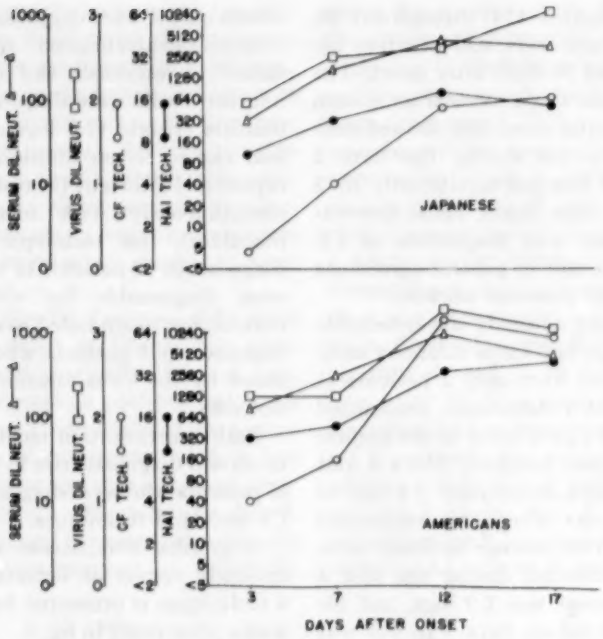

FIG. 2.—Comparison of average antibody response as detected by four techniques following Japanese B encephalitis.

but remained above 2.0 logs, and in the other patient there was no significant change.

Studies of 190 Japanese schoolchildren to be presented in the next paper of this series[1] indicated that persistence of HAI titers in excess of 1:40 one year after infection is unusual although a 1:10 titer may remain detectable for 5 to 8 years. Survey studies of adult Japanese and Koreans[9] gave similar results. These facts, together with the observed lower maximum HAI titers in Japanese as compared to Caucasians, suggest that in Japanese patients titers of 1:160 would be highly suggestive of current infection even in the absence of demonstrable rise in titer.

TABLE 2.—*Persistence of antibody one year after Japanese B encephalitis.*

Technique	Patient	Maximum titer, days 10–60	Titer at: 12 months	Titer at: 15 months
HAI	G.A.	1:640	1:160	1:40
	S.V.	1:2560		1:320
CF	G.A.	1:16	<1:4	<1:4
	S.V.	1:64		1:4
Virus dilution neutralization	G.A.	4.4 logs	2.3 logs	
	S.V.	3.2 logs		3.8 logs

The foregoing analysis considers only those cases in which the diagnosis of Japanese B encephalitis was established by CF criteria. It is of importance to determine whether any cases could be diagnosed by the HAI technique when *not* diagnosed by CF. For this purpose those patients are considered who were categorized as "presumptive Japanese B encephalitis" on the basis of positive HAI tests without diagnostic CF tests. All 20 patients in this category were Japanese. Eight patients had CF titers of 1:4 or 1:8 but had either no rise or only a 2-fold rise in CF titer in

the serums available for study. Six of these had diagnostic HAI tests (titer of 1:640 or greater, or 4-fold rise in titer). The other two had maximum HAI titers of 1:160 and 1:320, which is highly suggestive of recent infection in a Japanese patient (see above). Twelve patients had negative CF tests (at 1:4) in all specimens but had HAI titers considered diagnostic by the above criteria. Chanock et al[9,11] demonstrated that infection by certain viruses (West Nile, Murray Valley Encephalitis, St. Louis, and dengue) produces antibody which cross-reacts with JBE virus antigen in the HAI test. However, since these cross-reacting viruses apparently do not exist in Japan it seems safe to assume that the patients in this "presumptively diagnostic" category actually had JBE virus infection which was diagnosed by HAI but not by CF test. If this be true, the HAI test established diagnosis in approximately 30% more patients than did the CF test. It should be noted that these patients who were diagnosed by HAI but not by CF were not necessarily poor antibody formers. Diagnosis by CF criteria was impossible in many cases because serums were not obtained late enough after onset, or occasionally because the volume of serum submitted for analysis was so small that serial studies by CF technique were not possible. This analysis concerns, then, not the ability of the patient to form antibody, but the ability of the laboratory to reach a diagnosis under the less than ideal conditions of actual medical practice.

DISCUSSION

These data demonstrate that the HAI technique is uniquely valuable for the rapid diagnosis of JBE virus infection, confirming the results and conclusions of Chanock et al.[9,10] When the technique is established as a laboratory routine it is inexpensive and can be completed within 48 hours of receipt of serum. It permits diagnosis on the basis of a rising titer during the first 10 days of illness in many patients. Furthermore, in the absence of a demonstrable rise diagnosis can be confidently made on the basis of absolute antibody titers of 1:640 or greater (probably 1:160 in Japanese patients), since titers of this magnitude have not been found to persist from infections contracted in previous years. In tropical climates where JBE virus may occur in endemic, rather than epidemic pattern (e.g., Philippines, Malaya), this later criterion could not be accepted for diagnosis. In areas where antigenically related viruses such as West Nile, Murray Valley encephalitis, and St. Louis viruses occur, definitive diagnosis necessitates confirmatory studies by the more specific complement-fixation technique. In contrast to the HAI technique, the CF technique may not detect antibody rise until the 3rd or 4th week after onset, and some patients may never develop sufficient CF antibody for diagnosis. In normal clinical practice diagnoses may be missed by CF technique because sera are not collected late enough after onset. Dickerson et al[4] have also stressed the high percentage of patients in whom CF antibody rise occurs between 4 and 8 weeks after onset of illness. The virus dilution neutralization technique suffers several defects as a diagnostic method. The very promptness of antibody response precludes demonstration of rising titer in most cases. No absolute titer for diagnosis can be accepted because of long persistence of neutralizing antibody level after prior infection. The test cannot be interpreted for at least 7 to 10 days after mice are inoculated and, like all in vivo techniques, it is expensive. The serum-dilution neutralization technique may be more satisfac-

tory for diagnosis of recent infection than is the virus-dilution test but is slower, more expensive, and less accurate than either of the in vitro tests.

Because of the long persistence of neutralizing antibodies, the neutralization test (virus dilution) is the most sensitive test for detecting serologic evidence of past infection. However since HAI antibody remains detectable at low titers for several years after infection this cheaper and faster test may be applicable in some survey studies especially those utilizing serums from children.

SUMMARY AND CONCLUSIONS

Antibody titers were determined in serial serum specimens from 23 American and 43 Japanese patients with a confirmed diagnosis of Japanese B encephalitis. The serological techniques were hemagglutination-inhibition (HAI), complement-fixation (CF), virus dilution neutralization, and serum dilution neturalization. For each of these serological techniques comparative time-titer curves were determined which indicate time of (1) appearance of detectable antibody, (2) achievement of maximum titers, (3) decline of titer, and (4) disappearance of detectable antibody. From these data the following generalizations are made.

CF antibody titers rose rather slowly, remaining undetectable in most patients during the 1st week of illness, and occasionally remaining undetectable during the 3rd week. In most patients maximum titers were achieved during the 3rd week of illness. Titers stayed at or near maximum for at least 2 months thereafter but dropped to a diagnostically insignificant level by 5 months.

Neutralizing antibody as measured by the virus dilution method appeared in high titer during the 1st week after onset of illness, increased only slightly thereafter, but persisted at high titers for over a year. The serum dilution netralization studies showed a similar antibody response curve, but in a few patients the rise was less abrupt than by the virus dilution technique, and limited data suggest that the titer may decrease after a few months.

HAI antibody became detectable in almost all patients within one week after onset of illness, reached maximum titer by two weeks, dropped sharply after 5 to 6 months, but remained detectable at low titers for several years.

Under the conditions of this study an HAI titer of 1:640 was diagnostic of current JBE virus infection even in the absence of a demonstrated rising titer. Titers of 1:160 and 1:320 were highly suggestive but could not be accepted as diagnostic because occasionally they may persist as residual from earlier infection. The HAI technique confirmed all diagnoses based on CF tests (66 patients) and established diagnosis on an additional 18 patients on whom diagnosis was not established by CF tests. The HAI technique gave diagnostic results faster than CF or neutralization techniques and was practical for routine use in a virus diagnostic laboratory.

Americans averaged higher antibody titers than Japanese by all except the serum dilution neutralization technique. This difference was marked and showed a high degree of statistical significance with the HAI technique. The cause of this difference was not learned.

SEROLOGICAL STUDIES OF ENCEPHALITIS IN JAPAN

II. INAPPARENT INFECTIONS BY JAPANESE B ENCEPHALITIS VIRUS

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The principal objective of this study was to detect inapparent infections by Japanese B encephalitis (JBE) virus in order to compare the antibody response with that which followed overt encephalitis and to estimate the comparative frequency of overt and inapparent infections. This was accomplished by surveying a group of Japanese school children for serum antibodies just prior to the summer encephalitis epidemic to detect nonimmunes, and repeating these studies in the nonimmunes after the epidemic season to detect antibody appearance. The preliminary survey provided data concerning applicability of the hemagglutination-inhibition (HAI) technique to survey work, and patterns of antibody distribution by age, sex, residence, and sibling relationships. These data will be presented first.

SEROLOGICAL SURVEY

Serological methods are outlined in the first paper of this series.[1] Serums were collected between June 23 and July 23, 1954 from 190 Japanese elementary school children (ages 8 to 11) living in the towns of Tsuruse, Yanase, and Miyoshi in Iruma-gun, Saitama Prefecture, Japan. This region adjoins the northwestern border of Tokyo-to. It contains rural villages of 2000 to 5000 population, and the intervening land is used for rice paddies, truck gardens, and small wood lots. None of the children in this study had a past history of central nervous system disease. The July collection of serums preceded the encephalitis season by at least 4 weeks as judged by concurrent studies of wild birds in this same region[2] and public health statistics from Tokyo-to.[3]

All serums were tested for antibodies against the Nakayama strain of JBE virus by the HAI technique starting at a serum dilution of 1:5. Most of the serums, including all of the HAI-negative specimens, were also tested by neutralization technique to determine the validity of the HAI test as a screening method. These comparative data are presented in table 1.

Every one of the 41 children who had detectable HAI antibody had a positive neutralization test (1.7 logs or greater). Conversely, every child with a negative neutralization test (less than 1.7 logs) was also negative by HAI. However, there were 10 children who had positive neutralization tests but negative HAI tests. Thus in this group the HAI test gave 12% false negative results but no false positives. These results indicate

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1. Southam, C. M. 1956, Serologic studies of encephalitis in Japan. I. Hemagglutination-inhibiting, complement-fixing, and neutralizing antibody following overt Japanese B encephalitis. J. Infect. Dis. 99: 155–162.

2. 406th Medical General Laboratory, U. S. Army. 1955, Annual Report for 1955.

3. Nihon noen, Tokyo-to. (Japanese B encephalitis in Tokyo-to). 1954, Prepared by the Tokyo Municipal Health Department. 49 pp.

TABLE 1.—*Comparison of HAI and neutralization techniques in June–July bleedings.*

Neutralization test	HAI results			
	Negative 1:5	Positive at 1:5–1:20	Positive at 1:40 and 1:80	Positive over 1:80
Negative 0.9 logs or less	69	0	0	0
Borderline (negative) 1.0–1.6 logs	5	0	0	0
Positive 1.7 logs or more	10	20	21	0

that the HAI technique can be used as a rapid and inexpensive screening test which, if positive, is a reliable indicator of antibody, and presumably of immunity, but that a person with no antibody must be further tested by neutralization technique before he can be considered nonimmune.

The occurrence of detectable antibody to JBE virus in this entire group of school children is summarized in table 2, with breakdown by age and sex. The occurrence of positive neutralizing antibodies was computed by assuming that all children with HAI antibodies also had neutralizing antibody and then adding those patients with negative HAI titers who had positive neutralization test. Sixty-two per cent of the 190 children had neturalizing antibody. The average age of males and females in this group is identical (9.8 years). The slight difference between sexes in percentage of antibody positives is not significant

TABLE 2.—*Antibody to Japanese B virus in Iruma-gun school children in June–July 1954.*

Group	Number of serum	Percent positive	
		Neutralization technique	HAI technique
Age 8	30	50	50
Age 9	40	57	50
Age 10	63	62	59
Age 11	57	67	61
All males	83	65	58
All females	107	59	55
Total	190	62	56

as judged by the chi-squared test ($X^2 = 0.6$). Percentage of positives increased progressively from 50% at age 8 to 67% at age 11. This is compatible with an average annual infection rate of between 9 and 10% of the nonimmune population. Assuming that the neutralization test detected all cases of past infection in these children, and that the average annual infection rate was 10%, it follows from the observed frequency of false negative HAI tests that HAI antibody remains detectable at titers of 1:5 for at least 5 and probably 8 years following infection.

Data were also analyzed according to location of residence. The areas were subdivisions of each town as established by the town government, with boundaries usually designated by roads and streams and including areas in the magnitude of 1 square mile. The number of children tested from each area ranged from 10 to 32. Among the 10 localities thus designated, the 3 with the lowest incidence of positive reactors were adjacent areas (Hongo, Shiro, and Sakanoshita) in the southeastern half of the town of Yanase. These 3 areas combined showed 45% positives among 53 children, while the lowest percent positive in any other one area was 50%, and all other areas combined had 68% positives. The average age of the children tested from southeastern Yanase was exactly the same as the total study group (9.8 years), thus excluding age difference as a factor. Inspection of these areas revealed no apparent differences in ecology (e.g., more paddies), which might explain the differences in frequency of past infections. Questioning of residents of these areas revealed that there had been no recent influx of population into the area and, specifically, the families which included nonimmune children were old residents. Application of the chi-squared test to

TABLE 3.—*Immunity to JBE virus in 10 pairs of siblings.*

	Observed		Theoretical (Random distribution)
	Number	Percent	Percent
Both siblings negative	2	20	14.4
Both siblings positive	3	30	38.4
One positive, one negative	5	50	47.1

the southeastern Yanase group vs. all other areas combined gave a chi-squared value of 6.7, indicating that although no explanation has been found it is improbable that the difference is due purely to chance.

Sibling relationships among the tested children were ascertained in one school from which two thirds of the total study group was obtained. There were 10 pairs of siblings. If antibody existed, i.e., if infection had occurred, in a purely random distribution among these 20 individuals the distribution of positives and negatives in these pairs would fit the formula $(a+b)^2$. The expected distribution based on a 62% antibody positive rate in the total study group is tabulated, together with the distribution actually found, in table 3. (Results of HAI and neutralization tests were in agreement on each of these 20 serums.) The fact that the observed distribution is what would be expected with a random exposure to infection is compatible with a mosquito dissemination of virus and would not be expected in a disease disseminated by person-to-person contact.

INAPPARENT INFECTIONS

During the period August 20 to October 6, 1954 encephalitis occurred in adjacent Tokyo-to in epidemic pattern (fig. 1), although the number of cases was small (355) as compared to the peak years of 1948 (1934 cases), 1950 (1182 cases), and 1952 (711 cases).

On December 21 and 23, 1954 repeat bleedings were obtained from 81 of these same school children, including 30 who, in their previous serums, had no detectable neutralizing or HAI antibody. Among these 30 children, 25 still had no detectable anti-JBE antibody and 5 (3 age 10 and 2 age 9) had become positive, an infection rate of 17% among the nonimmune population. This observed infection rate in this small study group is not inconsistent with the average yearly rate of 9 to 10% computed above.

Retrospective histories were obtained

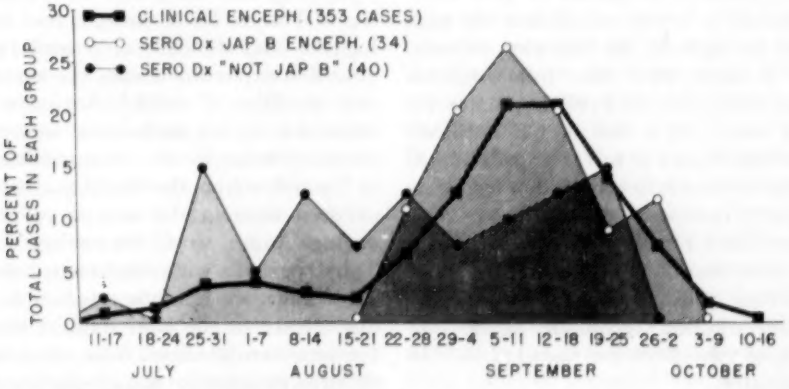

FIG. 1.—Distribution by time of onset of all clinical encephalitis, Japanese B encephalitis, and "encephalitis, not Japanese B," Tokyo 1954.

on each of the 5 school children who showed serological evidence of JBE virus infection. Four of them had no recognized illness in the interval between the two serum collections, had lost no time from school, and had played or worked every day during the 5-week summer vacation. The other child had a febrile illness with associated headache and 2 days of intermittent delirium which resulted in absence from school from 9 to 22 September. The diagnosis of encephalitis was considered for this child, but the final clinical diagnosis (according to the family) was merely fever of unknown origin. It seems highly probable that the JBE virus infection diagnosed by the serologic studies was the cause of this illness, which consequently merits the diagnosis of typical Japanese B encephalitis of moderate severity. No diagnosed case of encephalitis, and no other illness of severity comparable to the above occurred in the 3 schools where this study was conducted, according to the schoolmasters. The serologic findings on the 5 children in whom development of antibody occurred are presented in figure 2. The child who was ill is represented in this figure by open squares. Diagnosis of JBE virus infection is firmly established for each case by each of the following criteria: (1) a rise in HAI titer from negative (less than 1:5) to 1:10 or greater (4-fold rise); (2) a rise of CF antibody from less than 1:2 to 1:2 or greater; (3) a rise in neutralization index from less than 10 to more than 100 (logs of virus neutralized increased from less than 1 to more than 2); and (4) a rise in neutralizing antibody as measured by serum dilution technique against 25 LD_{50} of virus from less than 1:2 to 1:10 or greater.

A comparison of these serologic data

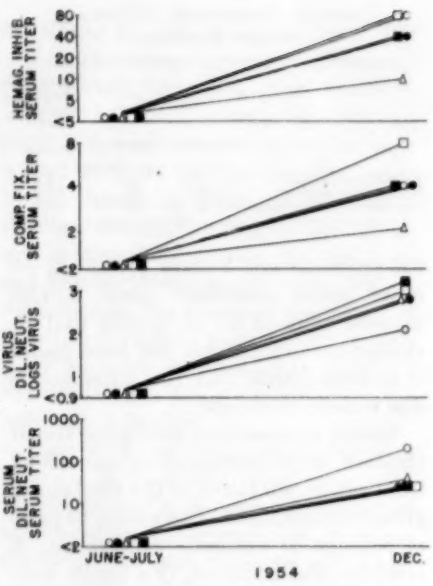

FIG. 2.—Antibody rise as demonstrated by 4 techniques following inapparent infection with Japanese B encephalitis virus. (Symbols distinguish individual children.)

with those seen following clinically diagnosed (overt) encephalitis is presented in figure 3. The data for overt encephalitis are those obtained from 44 Japanese residents of Tokyo of all ages and from 8 Japanese children of ages 8 to 11 residing in Tokyo. These patients had serologically and clinically diagnosed Japanese B encephalitis during the summer and autumn of 1954.[1] Antibody as measured by all techniques was consistently lower in the serums collected in December from the Iruma-gun school children than in the serums collected during August and September from Tokyo patients with overt encephalitis. This difference however appears to be due merely to the later date at which the sera were obtained from the school children rather than a quantitative difference in antibody production, since

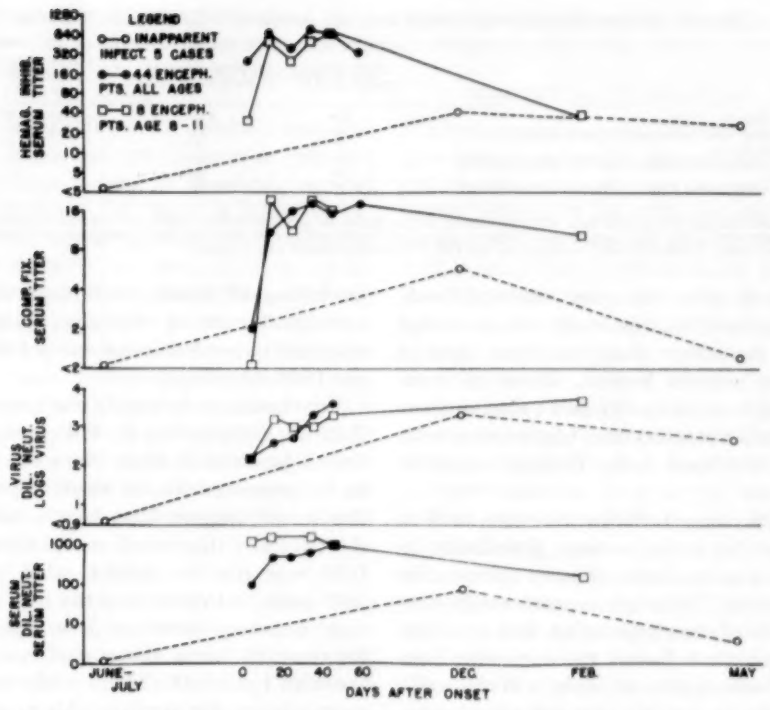

FIG. 3.—Comparison of average antibody response to overt and subclinical infection with Japanese B encephalitis virus.

the antibody titers of these two groups are similar in later serums.

It is of interest that 4 of the 5 school children who had JBE virus infection lived in that part of Yanase which was found, in the June–July serum survey, to have a significantly lower rate of antibody-positive children. The difference in infection rate between this and other regions might be due to chance (critical ratio 1.8), but it is clear that in 1954 there was at least as great a prevalence of virus in this region as in other parts of the study area. This occurrence of a lower rate of past infection and an equal or higher infection rate in 1954 in this area indicates, in the absence of human population change, that there must have been some recent ecological change in this area which has increased the prevalence of or transmission of virus.

Table 4 presents estimates of the ratio of overt to inapparent JBE virus infection based on the assumption that the findings in the Iruma-gun study group are representative of the entire student population of ages 8 to 11 in these same schools and on the further assumption that they are also representative of adjacent Tokyo. Applying the infection rate of 6% observed in the study group (5 infections among 81 children studied) to the entire 8- to 11-year-old population of these same 3 schools, it is estimated that 50 cases of virus infection occurred. The only diagnosed case of encephalitis in these

TABLE 4.—*Estimated relationship between overt and inapparent infection with JBE virus.*

	Study group (paired serums)	Students age 8–11 in 3 schools studied	Tokyo-to total population age 5–14
Total number children	81*	821*	1,581,812*
JBE virus infections (overt or inapparent)	5*	50	95,000
Clinical cases	1*	1*	161*
Ratio clinical encephalitis cases to total infections	1/5*	1/50	1/590
Deaths	0*	0*	49*
Ratio encephalitic deaths to inapparent infections	0/5*	0/50	1/1940

* Asterisks indicate factual data. All other figures are calculated on assumption that findings in the experimental group are applicable to the total population. Tokyo-to statistics in table and in text are from the mimeographed compilation, "Nihon Noen, Tokyo-to, 1954," compiled by the Tokyo Municipal Health Department.

schools was the case retrospectively diagnosed by this study. If we accept the statement that no other child in these schools had an illness of comparable severity, it follows that in these schools not more than 1 infection among 50 developed into clinically manifest disease.

Tokyo-to statistics were also used in order to study a large population in which an epidemic of overt encephalitis occurred. Although approximately two-thirds of this population live in urban or suburban Tokyo, the remainder lives in rural parts of Tokyo ecologically similar to and adjacent to the study area of Iruma-gun (Saitama Prefecture). The morbidity rate for Japanese B encephalitis in 1954 was of similar magnitude in urban and rural portions of Tokyo-to, so the assumption of a similar inapparent infection rate seems permissible. In the calculations for Tokyo-to it was necessary to use the 5 to 14 age group because statistics for smaller age divisions were not available. It seems probable that the inclusion of 11- to 14-year-old children with their lower percentage of nonimmunes (indicated by morbidity rates as well as by the above serological evidence) is more than balanced by inclusion of the 5- to 7-year-old children of greater susceptibility. Applying the observed infection rate of 6% to the Tokyo-to population of 5- to 14-year-olds, it is calculated that 95,000 cases of virus infection occurred. From this same population, 161 cases (including 49 deaths) were reported, a ratio of 1 case of clinically apparent encephalitis per 590 infections or 1 death per 1940 infections.

For these calculations it was assumed that all encephalitis in Tokyo-to was due to Japanese B virus. Since the data to be presented in the third paper of this series[4] suggest that nearly half of the clinically diagnosed encephalitis in 1954 was due to agents other than JBE virus, it follows that the true relation between overt and inapparent Japanese B virus infections may be closer to 1 per 1000. There is obviously great chance for error in this type of calculation since it is based on numerous assumptions and little experimental data, but in spite of this wide margin of error these calculations are acceptable as a first approximation. The fact that inapparent infection by JBE virus occurs frequently has been repeatedly demonstrated by serum surveys which indicate high frequency of neutralizing antibody titers in people with no history of encephalitis, but no previous study is known to the present author in which the time of and rate of serologic conversion has been observed and in which the antibody response to apparent and inapparent infections by JBE virus has been compared. This same approach to the study of inapparent infection has

4. Southam, C. M. 1956, Serologic studies of encephalitis in Japan. III. Epidemic encephalitis other than Japanese B encephalitis in Tokyo. J. Infect. Dis. 99: 170–173.

been used for poliomyelitis in the United States by Melnick,[5] who estimated 6 to 16 cases of paralytic poliomyelitis per 1000 infections.

None of those 51 children who had detectable antibody in June or July and who were re-bled in December showed an antibody rise. Since it would be expected that if infection by JBE virus had occurred in these children an increase in antibody would have occurred, it seems justifiable to conclude that reinfection did not occur among the children with previously detectable antibody. The difference in infection rate between these 2 groups (0% vs. 16%) is statistically significant (critical ratio, 2.5). It follows that the presence of circulating HAI antibody is indicative of clinical immunity.

In May of 1955 a third serum specimen was obtained from each of the five children who had developed antibodies during the summer. Antibody was still detectable in each child by HAI and both neutralization techniques at titers only slightly lower than in the December serums. Averaged data are included in figure 3. CF antibody remained detectable in only one child and at a titer of only 1:2.

5. Melnick, J. L. 1954, Application of tissue culture methods to epidemiological studies of poliomyelitis. Am. J. Pub. Health, **44**: 571–580.

SUMMARY AND CONCLUSIONS

Five cases of Japanese B virus infection were diagnosed in Japanese school children by study of paired serums collected before and after the encephalitis season. All but one of these children had a completely asymptomatic infection. Antibody response to inapparent infection was demonstrable by all techniques, and was similar in degree and duration to that which followed overt encephalitis. The demonstrated infection rate among susceptible children in 1954 in this study group was 17%. The antibody-positive rates for 8-, 9-, 10-, and 11-year-old children were 50, 57, 62, and 67%, respectively, indicating an average yearly infection rate of 9 to 10% among nonimmunes. There was no difference in antibody-positive rate or antibody titers between males and females. The occurrence of antibody in 10 pairs of siblings was consistent with a random distribution and hence consistent with mosquito transmission rather than contact transmission of the virus. It was estimated that at least 500 and possibly 1000 inapparent infections by Japanese B virus occurred per 1 case of clinically apparent disease.

SEROLOGICAL STUDIES OF ENCEPHALITIS IN JAPAN

III. EPIDEMIC ENCEPHALITIS OTHER THAN JAPANESE B ENCEPHALITIS IN TOKYO

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It is stated by Japanese doctors who participate actively in the clinical care and laboratory diagnosis of epidemic encephalitis in Tokyo that in the early weeks of an epidemic many patients fail to develop antibodies diagnostic of JBE virus infection, but as the epidemic progresses serologic diagnosis is readily established in most cases. During the study of antibody reactions in patients with Japanese B encephalitis reported in the first paper of this series[1] a group of 40 Japanese patients were studied who had a clinical diagnosis of nonbacterial encephalitis but who showed no serologic evidence of recent Japanese B encephalitis (JBE) virus infection. The present paper is an analysis of this phenomenon.

Serological techniques have been previously described.[1] All patients considered in this paper were Japanese hospitalized in the municipal hospitals of Tokyo-to, and all were studied by both complement fixation (CF) and hemagglutination-inhibition (HAI) techniques with serial serum specimens covering a minimum period of 21 days after onset of illness. All diagnoses of Japanese B encephalitis were based on both CF criteria (4-fold rise in titer and /or titer of 1:16 or greater), and HAI criteria (4-fold rise and/or titer of 1:640 or greater). Diagnoses based on HAI criteria alone have been excluded from this study but their inclusion would not affect the conclusions. Patients diagnosed as *not* having JBE virus infection showed no detectable CF antibody (less than 1:4), no rise in HAI titer, and no HAI titer in excess of 1:40 in any specimen. About half of these patients had neutralizing antibody for JBE virus indicating prior infection by the virus. Several cases had to be excluded because date of onset could not be ascertained.

In figure 1 on page 165 three curves are presented. One of these (black squares connected by heavy line) concerns all 353 patients reported to the Tokyo-to health department as having a final clinical diagnosis of encephalitis from June through October 1954.[2] The points on the curve indicate what percentage of patients in this group had onet of illness during the indicated week. Another curve (open circles connected by narrow line) concerns 34 Japanese patients from Tokyo who were diagnosed as having JBE virus infection by serologic (CF and HAI) studies performed in this laboratory and whose

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1. Southam, C. M. 1956, Serologic studies of encephalitis in Japan. I. Hemagglutination-inhibiting, complement-fixing, and neutralizing antibody following overt Japanese B encephalitis. J. Infect. Dis. 99: 155–162.

2. Southam, C. M. 1956, Serologic studies of encephalitis in Japan. II. Inapparent infections by Japanese B encephalitis virus. J. Infect. Dis. 99: 163–169.

date of onset was known. The points on the curve are the percentage of patients in this group with onset of illness during the indicated week. The third curve (black circles connected by narrow line) represent 40 encephalitis patients from the same epidemic who showed no serologic evidence of JBE virus infection. Again, points on the curve indicate percentage of patients in this group with onset during indicated week. The 34 "proved Jap B" cases and the 40 "encephalitis, not Jap B" cases are all included in the total group of 353 "clinical encephalitis cases." Thus the cases studied serologically with serum specimens adequate for diagnosis and with stated date of onset represent 21% of the total group. Curves are plotted in terms of percentages for each group to permit easier comparison of time distribution.

These curves (fig. 1 on page 165) indicate that: serologically proved JBE virus infection coincided in time and distribution pattern with the *peak* of the encephalitis epidemic; during the first 6 weeks of the epidemic none of the cases studied in this laboratory showed serological evidence of JBE virus infection; and "encephalitis, not Jap B" occurred in fairly constant numbers throughout the entire epidemic period. The fact that the time distribution is so different for the "JBE" and "not JBE" groups indicates that failure to demonstrate anti-JBE antibodies is not a result of poor antibody-producing capacity of certain patients, because if such were the explanation a constant *proportion* of patients should be non-diagnosable at all times and the curves should coincide. Furthermore, lack of antibody response is not the result of a mild infection since even after clinically inapparent infection antibody response is demonstrable by all techniques.[3]

When the 2 groups of patients—"Japanese B encephalitis" and "encephalitis, not Jap B"—were classified as to severity of clinical illness it was apparent that they also differed in clinical characteristics. Table 1 indicates the clinical severity of illness, as reported by the clinicians, of all patients for whom such data could be obtained. Data from American patients with proved Japanese B encephalitis are included for comparison. Most of the Americans became ill in Okinawa. All Japanese were from Tokyo. All of the 20 Japanese and all but 2 of the 21 American patients who were serologically diagnosed as Japanese B encephalitis were classified as having typical encephalitis of mild or severe degree. In contrast, among 13 patients diagnosed as "encephalitis, not Jap B" only 1 had severe encephalitis, and the others had a mild or minimal or atypical encephalitic illness.

From the above data it seems clear

3. Nihon noen, Tokyo-to. (Japanese B encephalitis in Tokyo-to). 1954, Prepared by the Tokyo Municipal Health Department. 49 pp.

TABLE 1.—*Relationship between clinical severity of illness and serologic diagnosis. Figures indicate number of patients.*

	Total	Typical encephalitis			Minimal or atypical	Clinical diagnosis changed
		Sequelae	Severe	Mild		
Jap B encephalitis						
American patients	21	2	7	10	2	0
Japanese patients	20	4	4	12	0	0
All patients	41	6	11	22	2	0
Encephalitis, not Japanese B	13	0	1	5	4	3
Total	54	6	12	27	6	3

that there were 2 superimposed epidemics of non-bacterial encephalitis in Tokyo during the summer of 1954. One was the result of JBE virus infection. It caused typical encephalitic manifestations with a high incidence of sequelae and mortality. It occurred as an explosive epidemic between mid-August and mid-October with peak incidence in mid-September. The other was the result of an unidentified pathogen. It caused a milder illness and occurred with fairly steady incidence from mid-July through September. The fact that this analysis was undertaken because of reputedly similar serologic observations in previous years suggests that the evidence obtained in 1954 may represent a frequent or general phenomenon in this area.

The causative agent or agents of this second type of encephalitis is not known. The comparatively mild course of the disease and the possible lack of fatalities may explain why an etiologic agent has not been isolated. A bacterial etiology was excluded in most cases, presumably in all cases, by clinical course, spinal fluid findings, and bacteriological studies performed in the hospital laboratories. The clinical picture suggested viral infection. If a virus was the causative agent it probably is completely unrelated to JBE virus but conceivably could be a variant of JBE virus which lacks the complete antigenic composition. The possibility that the second type of encephalitis was poliomyelitis should be serologically investigated, but clinically this diagnosis seems unlikely. Similarly, it seems improbable that it represents "type A" (von Economo's) encephalitis because of its mild clinical picture. Since mumps virus, lymphocytic choriomeningitis virus, and leptospira sometimes cause central nervous system infections,[4,5] serums from patients with "encephalitis, not Jap B" were studied for antibodies against these agents. Complement-fixation tests with mumps viral antigen were performed with acute and convalescent paired sera from 11 of these patients, and convalescent sera only (serums collected at least 21 days after onset of illness) from another 7. These included 10 patients with no detectable antibody to Japanese B virus and 8 with serological evidence of prior JBE virus infection. None of the paired sera showed a 4-fold rise in antibody to mumps virus. Five of the 18 patients had titers of 1:32 to 1:128. These titers suggest mumps infection in recent months, but do not constitute evidence that such infection was the cause of the encephalitis, and are not unexpected in children of school age. Mumps CF titers were also determined on 9 convalescent sera from the "proved Japanese B encephalitis" group. One of these had a titer of 1:32 while all others were lower. These data indicate that mumps virus was not a common cause of encephalitis in Tokyo in 1954 but the possibility that it was an occasional cause is not excluded. Convalescent serums from 7 of the "encephalitis, not Jap B" patients were tested for complement-fixing antibody against lymphocytic choriomeningitis virus. All were negative at a serum dilution of 1:2. Similarly, convalescent sera from 6 of these patients were tested for agglutinating antibody to 16 strains of leptospira* and all were negative

4. Adair, C. V., Gauld, R. L. and Smadel, J. E. 1953, Aseptic meningitis, a disease of diverse etiology: clinical and etiologic studies on 854 cases. Ann. Int. Med. **39**: 675–704.
5. Meyer, H. M., Jr., Rogers, N. G., Dascomb, H. E. and Payne, F. E. 1955, Diagnosis of central nervous system infections with special reference to aseptic meningitis caused by poliomyelitis virus. Bact. Proc. p. 66.

* Leptospirosis agglutination-lysis tests were performed by the Department of Bacteriology of the 406th Medical General Laboratory.

(less than 1:100). The possibility of coxsackie virus infections was not investigated.

Since the abrupt cessation of Japanese B encephalitis epidemics with the onset of cold weather is explained by the disappearance of the mosquito vector and since this epidemic of undiagnosed illness ceased simultaneously with the Japanese B encephalitis epidemic, it seems possible that this illness too is transmitted by a summer insect.

SUMMARY AND CONCLUSIONS

Forty Japanese patients in Tokyo with a clinical diagnosis of Japanese B encephalitis during the summer of 1954 showed no serologic evidence of Japanese B virus infection in serial serum specimens collected during illness and convalescence. This illness was first reported in mid-July and persisted with a fairly constant incidence through September. In contrast, seroiogically diagnosable Japanese B encephalitis did not appear until the later part of August, and increased explosively in incidence to a peak in mid-September. Both illnesses ceased abruptly at the end of September. The "encephalitis, not Jap B" was a milder illness than Japanese B encephalitis. None of the patients in this series had severe encephalitis and none had neurological sequelae. The etiologic agent or agents of this illness were not determined. Limited serological studies gave no reason to believe that mumps virus, lymphocytic choriomeningitis virus, or leptospira were frequent causative agents of this disease.

COMPARATIVE STUDY OF HOST RESISTANCE OF GUINEA PIGS AND RATS

V. THE EFFECT OF PNEUMOCOCCAL PRODUCTS ON GLYCOLYSIS AND OXYGEN UPTAKE BY POLYMORPHONUCLEAR LEUCOCYTES

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The work reported here is a comparative study of the action of components of a test organism, type I pneumococcus, on the metabolism of polymorphonuclear leucocytes from a susceptible species, the white rat, and from a resistant species, the guinea pig. These investigations are part of a program intended to shed light on host factors which may be responsible for differences in resistance of different species of hosts to a given organism. Other phases of this program have been reported.[1–4]

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METHODS AND MATERIALS

Polymorphonuclear leucocytes were obtained from guinea pig and rat peritoneal exudates by the method of Nungester and Ames.[5] Anaerobic acid production and oxygen uptake by leucocytes under various conditions of the experiments were measured on the Warburg respirometer essentially as outlined by Umbreit, Stauffer and Burris.[6] Anaerobic conditions were established with a mixture of 5% CO_2 and 95% N_2. Oxygen uptake was measured in an air atmosphere. Mariñelarena[7] found the use of similar techniques satisfactory in studying the metabolism of leucocytes.

Leucocyte suspensions were standardized by counting the number of cells with a Spencer bright-line haemocytometer following the conventional procedure. From a series of replicate counts, using two pipettes and two chambers for each count, the error was estimated to be ±5%. These suspensions contained from 30 to 40 thousand cells per cubic millimeter, and 2 ml of suspensions of this concentration range were pipetted into the Warburg flasks for measurements of gas exchange. The number of leucocytes per flask ranged between 60 and 80 million cells. In this range of concentration of leucocytes, the maximum and minimum total gas exchanges measured were in the order of 400 and 75 cmm, respectively. Replicate gas exchange measurements on aliquots of a leucocyte suspension gave curves falling within a ±4% range. Each actual experiment was done in duplicate and repeated three times. All experiments of a group showed the same

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6. Umbreit, W. W., Burris, R. H. and Stauffer, J. F. 1949, Second Edition, Manometric Techniques and Related Methods for the Study of Tissue Metabolism, Minneapolis. Burgess Publishing Co.
7. Mariñelarena, R. 1950, The effects of various chemical substances and bacteria on the glycolytic and respiratory activities of leucocytes. Doctoral dissertation, Dept. of Bacteriology, Univ. of Michigan.

trend of change and the final stimulation or inhibition was in the same order of magnitude. A typical experiment from each group is given under results. Statistical analysis of such small numbers could contribute but little to the interpretation of the results. Since the work reported here is a comparative study, the interpretation depends on a comparison of the metabolic responses of guinea pig and rat leucocytes to pneumococcal components or conditions established in pneumococcal infection in each animal. From the ±5% error estimated in counting the cells and the ±4% error estimated in measuring gas exchange, the total experimental error of any given results obtained from a single manometer can be calculated with the formula: $X = \pm\sqrt{x^2+y^2}$; substituting the above values for x and y, the individual errors, then total error $X = \pm\sqrt{5^2+4^2} = \pm\sqrt{41} = \pm 6.4\%$. When responses are of a small magnitude, interpretation is difficult or impossible. However, when the difference between responses of rat and guinea pig leucocytes is large, this difference can safely be considered a true one.

Type I *Diplococcus pneumoniae*, Michigan strain no. 76, cultured in brain-heart infusion broth (BHI), was used as the test organism. Filtrates of in vivo cultures were prepared from peritoneal exudates induced by inoculating guinea pigs intraperitoneally with 80 ml Locke-glucose* solution and 0.8 ml of a 6-hour culture of the pneumococcus. In rats, 0.4 ml of culture and 40 ml of Locke-glucose solution were used. The exudates were harvested 5 hours after inoculation, centrifuged and the supernates filtered through an ultrafine sintered glass filter. Controls for experiments testing the effect of pneumococcus-induced exudates were aliquots of the same leucocytes suspended in filtrates of exudates prepared as above but with the pneumococcus inoculum omitted. The exudates during the preparation of the filtrates were kept at about 5 C. Purified type I capsular polysaccharide (SI) was prepared by F. C. Kull in this laboratory, according to the method of Heidelberger, McLeod, Markowitz and Roe.[8] The level of virulence of the pneumococcus for rats and guinea pigs was checked periodically by inoculating these animals intraperitoneally with various dilutions of a culture of the organism. Ten to 100 organisms consistently killed rats in from 18 to 48 hours. The lethal dose for guinea pigs under the same conditions was in excess of 10^8 organisms.

* NaCl, 0.9%; $CaCl_2$, 0.042%; KCl, 0.024%; $NaHCO_3$, 0.08%; glucose, 1.0%.

8. Heidelberger, M., MacLeod, C. M., Markowitz, H. and Roe, A. 1950, Improved methods for the preparation of the specific polysaccharides of pneumococcus. J. Exper. Med. 91: 341–349.

RESULTS

1. The effect of filtrates of in vivo pneumococcus cultures.—There was a marked difference between the effect of filtrates of homologous in vivo cultures of pneumococci on glycolysis by rat and guinea pig leucocytes as shown in table 1. A plot of these results is shown in figure 1. Glycolysis by guinea pig leucocytes suspended in filtrates of homologous pneumococcus-induced exudates was stimulated by 61% at 90 minutes and this dropped to 54% at 150 minutes. Rat leucocytes, on the other hand, showed a slight stimulation of 7% at 35 minutes which dropped to an inhibition of 36% at 150 minutes. Measurements of the same activity and under the same conditions but with glucose added to a concentration of 0.01 *M* showed a parallel effect but the difference between the effects on rat leucocytes and guinea pig leucocytes was less. From a stimulation of 18% at 15 minutes, glycolysis

TABLE 1.—*The effect of filtrates of in vivo cultures of virulent pneumococcus on glycolysis by guinea pig and rat leucocytes (temperature 37 C, pH 7.8).*

Time in minutes	Source of leucocytes	Control* cmm CO_2 / 10^8 cells	Test† cmm CO_2 / 10^8 cells	Percent difference from control‡
35	Guinea pig	1.18	1.30	10
	Rat	1.22	1.30	7
60	Guinea pig	1.54	2.20	43
	Rat	2.18	2.20	1
90	Guinea pig	1.88	3.02	61
	Rat	3.25	2.68	−18
120	Guinea pig	2.22	3.44	55
	Rat	4.40	3.08	−30
150	Guinea pig	2.46	3.79	54
	Rat	5.35	3.42	−36

* Leucocytes suspended in filtrate of Locke-solution-induced exudate.

† Leucocytes suspended in filtrate of pneumococcus-induced exudate.

‡ Difference from control as percent of control

$$= \frac{\text{test in cmm} - \text{control in cmm}}{\text{control in cmm}} \times 100.$$

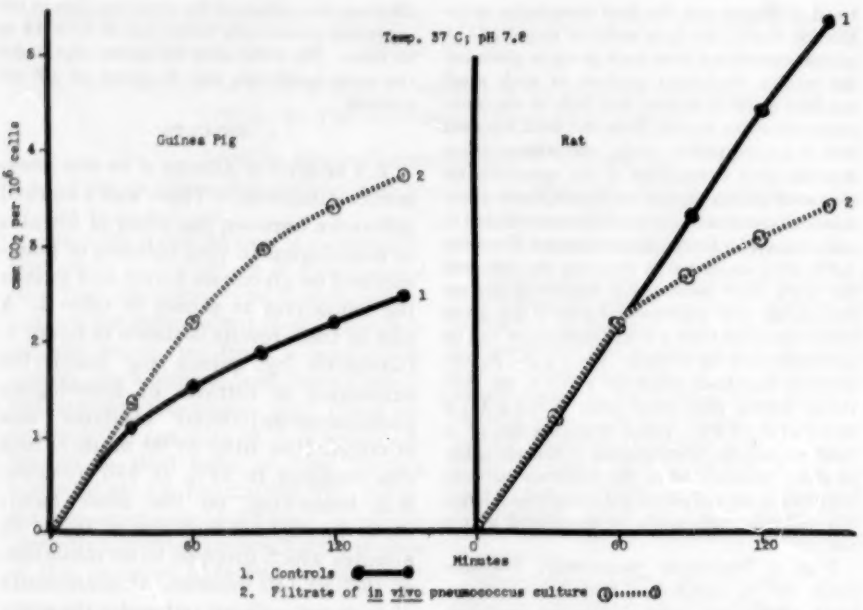

FIG. 1.—The effect of filtrates of in vivo cultures of virulent pneumococcus on glycolysis by guinea pig and rat leucocytes.

by guinea pig leucocytes dropped slowly so that total glycolysis was equal to that of the control at 135 minutes. Rat leucocytes showed an inhibition of 9% and this increased to 20% at the same respective time periods. Similar experiments with pyruvate added as a substrate resulted in a 21% stimulation of glycolysis by guinea pig leucocytes at 135 minutes, the end of the experiment. At this same time, glycolysis by rat leucocytes was depressed by 25%. The results of the experiments with glucose or pyruvate as added substrate are summarized in table 2, and the results with pyruvate are also plotted in figure 2.

TABLE 2.—*The effect of filtrates of in vivo cultures of pneumococcus on glycolysis by guinea pig and rat leucocytes with glucose or pyruvate as added substrate (temperature 37 C, pH 7.8).*

Time in minutes	Source of leucocytes	0.01 *M* glucose			0.01 *M* pyruvate		
		Control* cmm CO_2 / 10^6 cells	Test† cmm CO_2 / 10^6 cells	Percent difference from control‡	Control* cmm CO_2 / 10^6 cells	Test† cmm CO_2 / 10^6 cells	Percent difference from control‡
15	Guinea pig	0.55	0.65	18	0.99	1.09	10
	Rat	0.57	0.52	−9	0.93	0.92	−1
45	Guinea pig	1.73	1.87	8	1.57	2.22	41
	Rat	1.70	1.40	−18	2.00	2.00	0
75	Guinea pig	2.81	2.90	3	2.06	2.70	31
	Rat	2.72	2.28	−16	2.84	2.24	−21
105	Guinea pig	3.80	3.82	1	2.52	3.15	25
	Rat	3.64	3.00	−19	3.64	2.64	−27
135	Guinea pig	4.60	4.60	0	2.94	3.56	21
	Rat	4.44	3.57	−20	4.08	3.04	−26

*,†,‡ See footnotes, table 1.

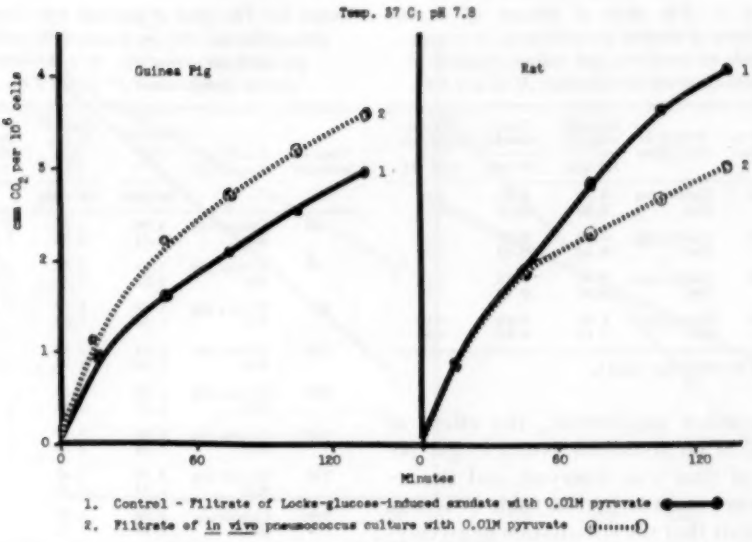
Temp. 37 C; pH 7.8
Guinea Pig
Rat
cmm CO_2 per 10^6 cells
Minutes
1. Control - Filtrate of Locke-glucose-induced exudate with 0.01M pyruvate
2. Filtrate of in vivo pneumococcus culture with 0.01M pyruvate

FIG. 2.—The effect of filtrates of in vivo cultures of pneumococcus on glycolysis by guinea pig leucocytes with 0.01 *M* pyruvate as added substrate.

Abels et al[9] were unable to demonstrate pyruvic acid decarboxylase in leukemic and normal human leucocytes. These authors demonstrated the conversion of pyruvate to lactic acid. However, Alonso[10] observed endogenous decarboxylase activity in rat and guinea pig leucocytes which was stimulated to a great extent by exogenous pyruvate in the case of guinea pig leucocytes, and to a very small degree in the case of rat leucocytes. Meyer and Levene[11] demonstrated the production of lactic acid from glucose by dog leucocytes. Presumably this took place via pyruvate. However, the degree of action of leucocytes on exogenous pyruvate could depend on the degree of permeability of the leucocyte to pyruvate.

Oxygen uptake by cells from both species of animals was inhibited when suspended in the respective filtrates with succinate (0.01 *M*) added as substrate. However, the degree of inhibition of the rat cells was greater than that of guinea pig cells. The results of this experiment are summarized in table 3.

2. The effect of purified type I capsular polysaccharide (SI).—This substance had a markedly different effect on glycolysis by rat and guinea pig leucocytes. The results, summarized in table 4, show that 1% SI stimulated glycolysis by guinea pig leucocytes by 8%, while glycolysis by rat leucocytes was stimulated by 139% at the end of 60 minutes. A concentration of 0.1% SI stimulated glycolysis by guinea pig and rat leucocytes by 8 and 41%, respectively.

9. Abels, J. C., Jones, F. L., Craver, L. F. and Rhoades, C. P. 1944, The metabolism of pyruvate by normal and leukemic white cells. Cancer Res. **4**: 149–152.
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TABLE 3.—*The effect of filtrates of in vivo cultures of virulent pneumococcus on oxygen uptake by leucocytes with sodium succinate as added substrate (temperature 37 C, pH 7.8).*

Time in minutes	Source of leucocytes	Control* cmm O_2 / 10^8 cells	Test† cmm O_2 / 10^8 cells	Percent difference from control‡
30	Guinea pig	0.26	0.22	−15
	Rat	0.30	0.25	−17
70	Guinea pig	0.60	0.52	−13
	Rat	0.68	0.53	−22
100	Guinea pig	0.86	0.75	−13
	Rat	0.98	0.79	−19
130	Guinea pig	1.07	0.94	−10
	Rat	1.19	0.95	−20

*,†,‡ See footnotes, table 1.

In another experiment, the effect of 0.1% SI on glycolysis over a longer period of time was observed, and the results are summarized in table 5. It will be noted that the stimulation of glycolysis by guinea pig leucocytes at 60 minutes is 1% and that of rat leucocytes is 31%, which are comparable to the values obtained in the previous experiment (table 4). The rates of glycolysis over a 4.5-hour period were calculated from the data in table 5. Figure 3 shows the curves obtained from the plotting of the rates of glycolysis. The rate for the 5th hour was estimated from the 4.5-hour value. These curves clearly demonstrate the difference in action of SI on guinea pig and rat leucocytes. The rate of glycolysis by guinea pig leucocytes gradually increased over that of the control during the entire 4.5 hours of observation. On the other hand, SI strongly stimulated glycolysis by rat leucocytes in the early period and then the rate of glycolysis decreased sharply so that it was equal to that of the control at about 170 minutes. From this point the rate of glycolysis continued to drop at a greater rate than that of the control.

TABLE 5.—*The effect of purified type I capsular polysaccharide (SI) on glycolysis by guinea pig and rat leucocytes in a 4.5-hour period (temperature 37 C, pH 7.4).*

Time in minutes	Source of leucocytes	Control* cmm CO_2 / 10^8 cells	Plus SI 0.1% cmm CO_2 / 10^8 cells	Percent difference from control†
60	Guinea pig	1.04	1.05	1
	Rat	0.71	0.93	31
90	Guinea pig	1.45	1.56	8
	Rat	1.07	1.34	25
120	Guinea pig	1.80	1.99	11
	Rat	1.34	1.63	22
150	Guinea pig	2.14	2.42	13
	Rat	1.63	1.92	22
180	Guinea pig	2.50	2.84	14
	Rat	1.88	2.14	18
210	Guinea pig	2.81	3.17	13
	Rat	2.06	2.29	11
240	Guinea pig	3.10	3.49	13
	Rat	2.24	2.42	8
270	Guinea pig	3.24	3.77	16
	Rat	2.44	2.51	3

*,† See footnotes, table 4.

3. The effect of molecular components of SI on glycolysis by guinea pig and rat leucocytes.—The components of SI are galacturonic acid and either glucosamine and acetyl, or acetylglucosamine. There are conflicting reports in the literature as to how the acetyl is combined with

TABLE 4.—*The effect of purified type I capsular polysaccharide (SI) on glycolysis by guinea pig and rat leucocytes in a 1-hour period (temperature 37 C, pH 7.4).*

Time in min. after addition of SI	Source of leucocytes	Control* cmm CO_2 / 10^8 cells	Plus SI 1% cmm CO_2 / 10^8 cells	Percent difference from control†	Plus SI 1% cmm CO_2 / 10^8 cells	Percent difference from control†
30	Guinea pig	0.53	0.58	9	0.57	6
	Rat	0.21	0.58	171	0.30	43
60	Guinea pig	1.05	1.13	8	1.13	8
	Rat	0.41	0.97	139	0.58	41

* Leucocytes in Ringer-$NaHCO_3$ solution.

† Difference from control as percent of control $= \frac{\text{test in cmm} - \text{control in cmm}}{\text{control in cmm}} \times 100.$

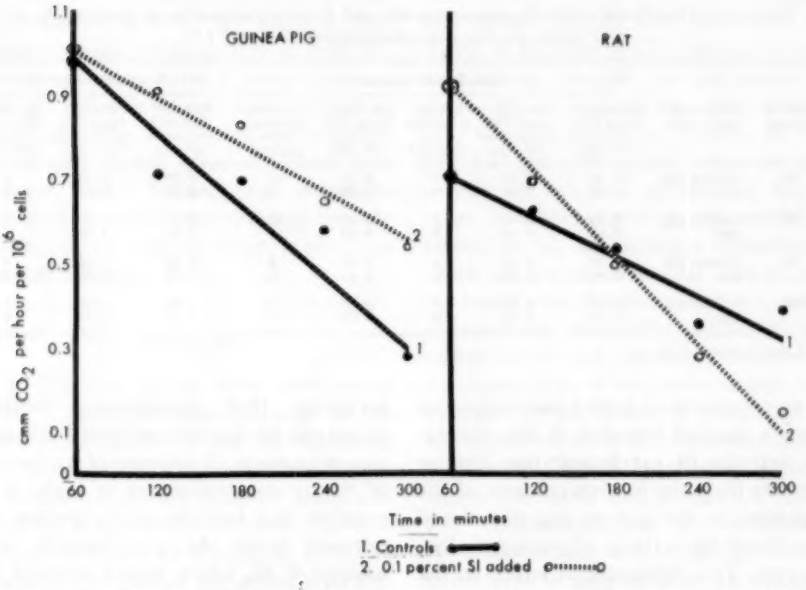

FIG. 3.—Change in rates of anaerobic acid production by guinea pig and rat leucocytes with 0.1% pneumococcus SI in a Ringer-bicarbonate suspending medium. Temperature 37 C, pH 7.4.

the rest of the SI molecule.[12–14] The results obtained with intact SI indicated that the effects of the various components of this substance should be investigated. Alpha-D-Na-galacturonate was prepared from alpha-D-galacturonic acid by neutralization with 0.1 *N* NaOH. Sodium galacturonate in 0.1 and 0.5% concentrations had but a slight stimulatory effect of glycolysis by either rat or guinea pig leucocytes. Similarly, glycolysis by these cells was slightly stimulated by N-acetylglucosamine. The results are summarized in table 6. In contrast to N-acetyl glucosamine and galacturonate, D-glucosamine-HCl had a strong inhibitory effect on glycolysis by both guinea pig and rat leucocytes. With cells from both species of animals the inhibition was quite high at 15 minutes and dropped somewhat in the case of guinea pig leucocytes and increased slightly in the case of rat leucocytes. The results obtained with glucosamine-HCl are summarized in table 7.

12. West, W. S. and Todd, W. R. 1951, Textbook of Biochemistry, New York, Macmillan Co.
13. Evans, T. H. and Hibbert, H. 1946, Bacterial polysaccharides. Advances in Carbohydrate Chemistry, vol. 2, New York, Academic Press, pp. 204–233.
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DISCUSSION

The type and level of activity of enzymes of phagocytic cells whose substrates are bacterial products or components would be of importance in the early phases of infection. A lowering of the efficiency of the metabolic reactions of these cells concerned with the yielding of energy would impair their ability to phagocytize and dispose of invading organisms. The results of the experiments reported above using filtrates of

TABLE 6.—*The effect of alpha-D-galacturonic acid and N-acetylglucosamine on glycolysis by rat and guinea pig leucocytes (temperature 37 C, pH 7.4).*

Time in minutes	Source of leucocytes	Alpha-D-galacturonate					N-acetylglucosamine		
		Control* cmm CO_2 / 10^8 cells	0.1%* cmm CO_2 / 10^8 cells	Percent difference from control†	0.5%* cmm CO_2 / 10^8 cells	Percent difference from control†	Control* cmm CO_2 / 10^8 cells	0.1%* cmm CO_2 / 10^8 cells	Percent difference from control†
30	Guinea pig	0.20	0.20	5	0.24	15	0.59	0.57	3
	Rat	0.26	0.23	9	0.23	9	0.57	0.63	11
60	Guinea pig	0.41	0.43	2	0.47	15	0.97	1.02	5
	Rat	0.46	0.43	7	0.43	7	1.03	1.07	4
90	Guinea pig	0.60	0.62	3	0.65	8	1.44	1.50	4
	Rat	0.66	0.68	3	0.62	6	1.44	1.49	3
120	Guinea pig	0.80	0.84	5	0.87	9	1.80	1.89	5
	Rat	0.85	0.84	0	0.85	0	1.74	1.84	6

*,† See footnotes, table 4.

in vivo cultures of type I pneumococcus show a marked lowering of the glycolytic activity of rat leucocytes. Similar filtrates from the in vivo cultures of the organism in the guinea pig stimulated the glycolytic activity of guinea pig leucocytes. This difference in effect on the leucocytes from the susceptible and resistant animals was also evident when either glucose or pyruvate was added as substrate. Analysis of the two different filtrates would have to be made to determine the substance or substances produced in each case that were responsible for the effects observed. The differential effect of SI on glycolysis by rat and guinea pig leucocytes may be explained by a difference in the rate of hydrolysis of SI and the subsequent observation that glucosamine inhibits glycolysis by leucocytes from both species of animals. If the rate of hydrolysis of SI by rat leucocytes is high, it is possible that initially acidic groups are released from the galacturonic acid moiety of SI, which would account for the increased CO_2 release from the bicarbonate buffer. The continuation of this hydrolytic process could result in the release of glucosamine, which at some point would reach an inhibitory concentration. The net result would then be that observed in the experiments testing the effect of SI on rat leucocytes over a 270-minute period. A low rate of hydrolysis of SI by guinea pig leucocytes would explain the low order of CO_2 release from bicarbonate buffer by these cells. A low hydrolysis rate of SI would also account for the inability of the system to achieve an inhibitory concentration of glucosamine. It must be added that to our knowledge no direct evidence for the hydrolysis of SI by leucocytes has been reported. No definite conclusions can be reached regarding the contributions of the metabolic differences between rat and guinea pig leucocytes to the relative resistance of these animals to type I pneumococcus. However, it is suggestive that in a number of conditions to which the leucocytes were subjected, the metabolic ac-

TABLE 7.—*The effect of D-glucosamine hydrochloride on glycolysis by guinea pig and rat leucocytes (temperature 37 C, pH 7.4).*

Time in minutes	Source of leucocytes	Control* cmm CO_2 / 10^8 cells	0.01 *M* glucosamine-HCl cmm CO_2 / 10^8 cells	Percent difference from control†
15	Guinea pig	0.50	0.09	−82
	Rat	0.23	0.11	−52
45	Guinea pig	1.06	0.23	−78
	Rat	0.64	0.24	−63
75	Guinea pig	1.57	0.33	−79
	Rat	0.97	0.35	−64
105	Guinea pig	2.12	0.48	−77
	Rat	1.27	0.49	−62

*,† See footnotes, table 4.

tivities of rat leucocytes measured were considerably lowered by various substances which are definitely or possibly associated with type I pneumococcus infection, while these same activities of guinea pig leucocytes were either stimulated or not affected. These conditions which lowered rat leucocyte metabolism could contribute to the low resistance of rats to type I pneumococcus infection.

SUMMARY

1. Filtrates of in vivo cultures of type I pneumococcus cultured in the peritoneal cavity of the rat markedly inhibited glycolysis by rat leucocytes. Similar results were obtained when glucose or pyruvate was added as substrate. Filtrates of similar in vivo cultures in the guinea pig stimulated glycolysis by guinea pig leucocytes under the same conditions of testing. Oxygen uptake by rat or guinea pig leucocytes was inhibited by the homologous filtrates with succinate added as substrate. The inhibition of the rat leucocytes was twice that of guinea pig leucocytes.

2. Glycolysis by rat leucocytes was stimulated for a short period of time and then inhibited by purified SI. Guinea pig leucocytes in a similar system showed a small and continuous increase in the rate of CO_2 evolution over that of the control. No inhibition of guinea pig leucocytes was observed.

3. Galacturonate, a moiety of SI, had a slight stimulatory effect on glycolysis by rat and guinea pig leucocytes. N-acetyl-glucosamine, possibly a moiety of SI, had a slight stimulatory effect on glycolysis by rat and guinea pig leucocytes. Another possible moiety, glucosamine, markedly inhibited glycolysis by rat and guinea pig leucocytes. An explanation of the initial stimulation followed by inhibition of glycolysis by rat leucocytes with SI is proposed on the basis of a high SI hydrolase activity which with time established an inhibitory concentration of glucosamine. A low SI-hydrolase activity by guinea pig leucocytes is suggested as an explanation of the small continous stimulation of glycolysis by guinea pig leucocytes in the presence of SI without ensuing inhibition.

4. It is suggested that the impairment of the metabolic functions of rat leucocytes by the pneumococcal substances tested may be a contributing factor to the low resistance of rats to type I pneumococcus infection.

STUDIES ON ACUTE RESPIRATORY ILLNESS IN NAVAL RECRUITS, WITH EMPHASIS ON THE ADENOVIRUSES (APC-RI)

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The isolation of adenovirus[1] (APC-RI) from military recruits with acute respiratory illness has been recently described.[2] The syndrome of "acute respiratory disease of recruits" (ARD)[3] was then shown to be associated with the adenovirus group.[4] Further studies have emphasized the frequency of the association of this group of viruses with respiratory illness in military recruit populations.[5–10]

The present report concerns studies of acute respiratory illnesses in Naval recruits occurring during the period September 1954 through August 1955.

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† Fellow of the National Foundation for Infantile Paralysis.

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MATERIALS AND METHODS

Population.—The present study was performed in the 10th Regiment of the Recruit Training Command, USNTC, Great Lakes, Illinois. The principal function of Recruit Training Command was to provide 9 weeks of indoctrination and training of newly inducted recruits for the Naval service. The men were between 17 and 22 years of age, with the majority coming from the midwest. A record of the number of recruits in this regiment was maintained on a daily basis. The average daily strength per week was used in computations of the respiratory illness admission rate.

Beginning in November 1954 one-third of the recruit population and all incoming recruits were inoculated with influenza polyvalent vaccine containing FW1-50A', FM1A' and Lee B viruses. The inoculation of incoming men was terminated on April 15, 1955 and observations for influenza continued throughout the period of study. Because of the high prevalence of streptococcal infections two 10-day periods of oral penicillin prophylaxis were initiated on March 3 and April 19, 1955.

Study procedures.—Personnel from the Naval Medical Research Unit No. 4 (NAMRU-4) were at the regimental dispensary to intercept all recruits coming to morning and noon sick call on weekdays and on Saturday morning. The men coming to sick call were seen by a hospital corpsman, who recorded their temperatures, chief complaints and took a nose and throat swab for isolation of β hemolytic streptococci.

A buffered medium, which was expectorated into 5% horse serum, was employed for collecting nasopharyngeal washings for virus isolations. Fifty ml vacuum tubes were used to obtain paired serums for antibody determinations. During the period of study both paired serums and nasopharyngeal washings on 280 men were taken. These individuals were irregularly selected from men with a respiratory illness of less than 48 hours duration and who were in their first 6 weeks of training. This allowed a 3 week convalescent blood to be obtained before their 9 weeks of training were completed.

Ninety-five of the 280 men were sufficiently ill with a respiratory illness to be admitted to the dispensary. Paired serums only were obtained from 918 additional recruits who were examined and admitted to the dispensary by a medical officer during their first 6 weeks of training. Thus, paired serums were obtained for serological study from 1013 recruits admitted to the dispensary with a febrile respiratory illness.

Laboratory methods.—As blood specimens were received at the laboratory, the serum was separated from the clot, frozen and stored at −24 C. Acute and convalescent specimens were paired and tested simultaneously.

The complement fixation test was performed according to methods previously described.[11,12] HeLa cell culture fluid infected with type 4 adenovirus was used as antigen in the complement fixation test. The adenovirus antigen was grown in 32 ounce prescription bottles. The HeLa cell growth medium (40% human serum and 60% Hank's solution) was washed from fully grown cultures and replaced by maintenance medium (5% horse serum* and 95% Mixture 199*). All mediums contained 100 units of penicillin and 0.1 mg streptomycin per ml. A 2 ml inoculum of stock virus was used and the supernatant fluid was harvested when +++ to ++++ cytopathogenesis appeared. Infected fluids were subsequently harvested and replaced daily as long as cells were present. The harvested fluids, which were inactivated by heat (56 C for 1 hour) served as complement fixing antigen. A 1:10 dilution of antigen was usually employed in the test. An antibody titer rise of two tubes or more, between the acute and convalescent serums, was interpreted to be a significant change.

Nasal washings were received at the laboratory within an hour after collection. The specimens were immediately homogenized by treatment in a power blendor for 2 minutes, divided in 1 ml aliquots and frozen and stored at −65 C.

The technic used for the isolation and identification of influenza viruses has been described elsewhere.[12] HeLa cell tube cultures (16 mm screw capped tubes) were employed for adenovirus isolation. One-tenth ml of nasal washing was added to each of three tube cultures on maintenance medium. The tubes were inspected every other day for evidence of cytopathogenic changes, through at least 14 days of incubation. The cytopathogenic agents isolated were typed by mixing 0.3 ml of supernatant fluid with 0.15 ml of heat

11. Rosenbaum, M. J. and Woolridge, R. L. 1955, The use of soluble antigen for the serological diagnosis of influenza in a vaccinated population. Research Project Report NM 005 051.06.07, the Naval Medical Research Unit No. 4., USNTC, Great Lakes, Ill.
12. Woolridge, R. L., DeMeio, J. L., Whiteside, J. E. and Seal, J. R., 1955, Epidemic influenza B and C in Navy recruits, 1953–54. I. Antigenic studies on influenza, type B. Proc. Soc. Exper. Biol. & Med. **85**: 430–435.

* Microbiological Associates, Inc., Washington, D. C.

inactivated type specific rabbit antiserum (in a 1:30 dilution) and incubating at 37 C for 30 minutes. One-tenth ml of the incubated mixture was inoculated into replicated HeLa cell tube cultures. The antiserum specific for the virus completely inhibited cytopathogenesis at the time control tubes showed advanced changes.

The nose and throat cultures for the isolation of streptococci were obtained on dry swabs and streaked directly onto sheep blood agar plates containing 5% sheep blood. The methods for grouping and typing used in this laboratory have been previously described.[13]

RESULTS

The respiratory disease admission rates per thousand per week for the 10th regiment are shown in figure 1. The rates were low during the fall months of 1954. They began to increase in January 1955 and reached peaks in February and in April and were lower during the spring and summer months.

Complement fixation antibody titer rises for the adenovirus group in the paired serums from 1013 recruits who were hospitalized at the dispensary with a febrile respiratory illness are also shown in figure 1. From September through December 1954 less than 10% of the paired serums showed antibody titer rises. The percent of titer rises increased sharply in January to 52%, reaching a peak of 74% during the middle of February, and remained high throughout the rest of February and March. During the spring and summer

13. Personnel of the United States Naval Medical Research Unit No. 4. 1952, The use of triethylene glycol vapor for control of acute respiratory diseases in Navy recruits. II. Effect on acute respiratory diseases. Am. J. Hyg. 55: 215–229.

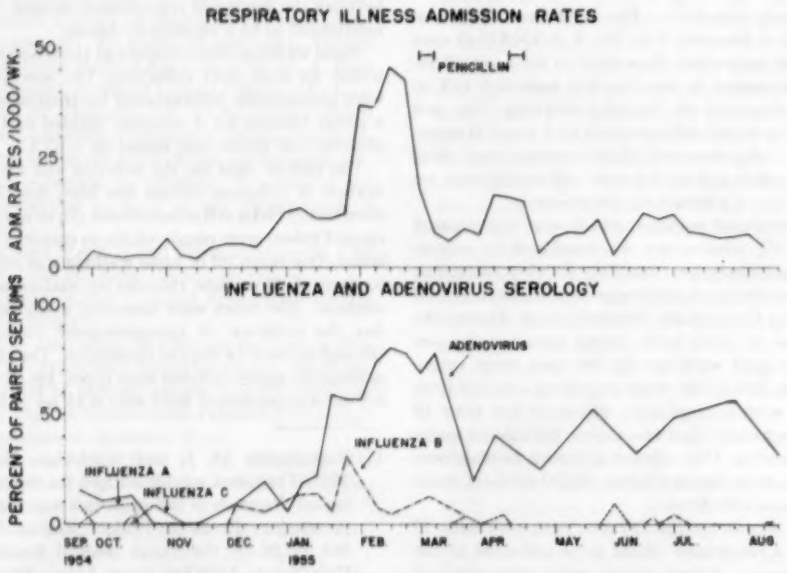

FIG. 1.—Acute respiratory illness admissions of Navy recruits to one regimental infirmary, Naval Training Center, Great Lakes, Illinois and complement fixing antibody titer rises to influenza and adenoviruses of men reporting to sick call. Data in the lower figure are given by the week in which the acute serums were collected. Each point represents a minimum of 20 serum pairs. A period of more than a week is covered in those instances where less than this number of serum pairs was available per week.

months the percent with titer rises remained high and several peaks of around 50% are evident. A total of 40% of the 1013 paired serums were found to have antibody titer rises.

Nasal washings (and paired serums) were obtained from 280 men reporting to the dispensary with a respiratory illness of 48 hours or less duration, in order to determine the prevalence of adenovirus types. The number and types isolated by month are shown in table 1. During the fall months of 1954 when the prevalence of respiratory tract infections was low only five adenovirus isolations were obtained (four type 3 and one type 5). Isolations of adenovirus type 7 were associated with the epidemic of respiratory illness in January and February, with a total of 29 strains of this type being identified during the first three months of 1955. In March four type 4 viruses were also isolated and thereafter only type 4 adenovirus was found. While no adenovirus was isolated in April, an additional 29 strains of type 4 virus were isolated from May through August. As seen in table 1, a total of 33 type 4 adenoviruses was isolated.

Fifty of the virus isolations came from the 95 recruits who were admitted to the dispensary, while only 17 adenoviruses were found in the nasal washings of the 185 men not admitted. All men from whom virus was isolated had a recorded temperature of 100 F or greater. The mean temperature in the group was 101.6 F. Symptoms recorded at the time the nasal washing was obtained showed that almost all recruits from whom adenovirus was isolated had sore throat (95%) and cough (91%). Many had nasal symptoms (75%), headache (70%) and chills (72%). Conjunctivitis was not seen. Four of the admitted men from whom adenovirus was isolated were found to have pneumonitis and were transferred to the base hospital with the diagnosis of primary atypical pneumonia. The rest of the admitted men had short illnesses with mean duration of fever of 2 days and were returned to duty in an average of 3 days.

TABLE 1.—*Number of nasal washings and adenovirus isolations from naval recruits reporting any respiratory illness.*

Month	Nasal washings	Adenovirus types			
		3	4	5	7
1954					
September	17	1			
October	36	3		1	
November	23				
December	13				
1955					
January	40				8
February	32		1		19
March	29		4		2
April	10				
May	7		4		
June	29		7		
July	21		12		
August	23		5		

Complement fixation antibody titer rises were demonstrated in 93 of the 280 pairs of serums. All men from whom a virus was isolated had a complement fixing antibody titer rise in their paired serums. The 26 recruits whose paired serums showed complement fixation antibody rises with adenovirus antigen but from whom viruses were not isolated had a mean temperature of 100.2 F and only 77% had sore throats.

In addition to the complement fixation rises, homologous neutralization antibody titer rises were found in all but two of these 67 serum pairs associated with virus isolation. Fifty pairs of these serums have been employed in studies on the development of a simplified neutralization test for adenovirus antibodies[14] and on the prevalence of hetero-

14. Grayston, J. T., Johnston, P. B., Smith, M. E. and Loosli, C. G. 1956. An improved technique for the neutralization test with adenoviruses in HeLa cell cultures. J. Infect. Dis. 99: 188–198.

typic responses to adenovirus types 1 through 10.[15]

The influenza complement fixation serology is also shown in figure 1. These data are from 798 men (of the 1013 men referred to above) who did not receive influenza vaccine in the 1954–1955 prophylaxis program. There were only a few antibody titer rises to influenza types A (GL1148-53); B (GL1760-54) or C (GL1167-54) from September through December and from February through August. During January there was an increase in the numbers of antibody titer rises to influenza type B. In the week ending 29 January, 9 of 32 (28%) paired serums had antibody titer rise to the type B antigen. Four influenza type B viruses were isolated from these men during this month. The viruses were found to be antigenically similar to the GL1738-54 type B virus.

Nose and throat cultures were taken from men reporting to sick call, regardless of their complaint, for the isolation of group A β hemolytic streptococci. Ninety-eight percent of the group A strains isolated were type 19. Streptococcal isolation rates reached a peak towards the end of February, at which time about 48% of the cultures were positive. The isolation rates declined rapidly when a 10-day period of 250,000 units *b.i.d.* of oral penicillin was given. When the isolation rates increased again in April, another 10-day period of penicillin prophylaxis was instituted. The recovery rates of streptococci were again reduced and remained low for the rest of the year. High isolation rates for group A streptococci were present in the personnel reporting to sick call whose primary complaint did not involve the upper respiratory tract during periods when streptococci were prevalent.

15. Grayston, J. T., Loosli, C. G., Johnston, P. B., Smith, M. E. and Woolridge, R. L. 1956. Neutralizing and complement fixing antibody response to adenovirus infection. J. Infect. Dis. **99**: 199–206.

DISCUSSION

Except for one week in January there is little evidence that influenza viruses were associated with respiratory illness of recruits at Great Lakes during this study. A considerable amount of illness was associated with β hemolytic streptococcal isolations. Penicillin prophylaxis reduced dispensary admission rates and isolation rates for streptococci. It was observed that all isolations of adenovirus were associated with febrile respiratory illnesses. The isolations were carried out on recruits at the dispensary and virus isolations were made from over 50% of the admissions as compared to less than 10% of the men not admitted. From this it may be inferred, despite the many administrative factors entering into decisions to admit, that adenovirus was associated with the relatively more severe upper respiratory illnesses. It is of interest that the 26 men showing complement fixing antibody titer rises to adenovirus from whom virus was not isolated experienced considerably less subjective illness. It is possible that adenovirus isolations can technically be best made when more severe illness is present, or it may be that some of these recruits suffered their adenovirus infection before or after the nasal washing was obtained but before the convalescent blood was drawn. Hilleman and associates[5,6] have reported that a significant percentage of recruits showing complement fixation antibody titer rise against adenovirus have not complained of upper respiratory illness.

In the year preceding these studies at Great Lakes, type 4 was the only adenovirus isolated.[10] Likewise, type 4 has been the only adenovirus recovered during the fall, winter and spring of 1955–56. Thus, the appearance of types 3, 5

and 7 during the fall and winter of 1954–55 is unusual as far as three years experience is concerned. The continued high respiratory admission rates during the spring and summer of 1955 was also unique for the Recruit Training Command at Great Lakes. As the above data indicate the high prevalence of respiratory infection during this period of time was due to adenovirus type 4. As has been the previous experience at Great Lakes, others[5,7,9] have found a marked decline in respiratory disease in military personnel during the summer months.

The finding that 40% of 1013 men hospitalized with febrile respiratory illness had serologic evidence of adenovirus infection cannot be interpreted that each of these men's symptoms were due to adenovirus. This is particularly true in this study because no comparable and extensive serologic testing was done in nonhospitalized recruits. However, the data from the 280 men along with the accumulating evidence in other studies[7–10,16,17] suggests the possibility that adenovirus infection caused a sizeable and serious amount of morbidity in the military recruit population.

16. Ward, T. G., Huebner, R. J., Rowe, W. P., Ryan, R. W. and Bell, J. A. 1955, Production of pharyngo-conjunctival fever in human volunteers inoculated with APC viruses. Science, **122**: 1086–1087.

17. Ginsberg, H. S., Gold, E., Jordan, W. S., Katz, S., Badger, G. F. and Dingle, J. H. 1955, Relation of the new respiratory agents to acute respiratory diseases. Am. J. Pub. Health. **45**: 915–922.

SUMMARY

Naval recruits at Great Lakes, Illinois, who were admitted to the dispensary with acute febrile respiratory illness during the year from September 1954 to August 1955, showed a 40% complement fixing antibody titer rise to adenoviruses. Serological evidence of infection by influenza, on the other hand, was found infrequently. Adenoviruses were isolated only from men with a febrile respiratory illness, almost all of whom had complaints of a sore throat. Fifty of the 67 adenovirus isolations were from 95 recruits who were admitted to the dispensary with a febrile respiratory illness, while the other 17 came from the 185 not admitted. Although types 3, 4, 5 and 7 were found during the year, types 4 and 7 were predominately responsible for the adenovirus infections. Rates of respiratory illness admissions remained relatively high during the summer months and adenovirus type 4 isolations, along with serological titer rises to adenovirus, continued throughout the summer. The data presented suggest that the adenoviruses and β hemolytic streptococci cause the majority of the more severe respiratory illnesses in the Naval recruits.

AN IMPROVED TECHNIQUE FOR THE NEUTRALIZATION TEST WITH ADENOVIRUSES IN HELA CELL CULTURES

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The recently discovered adenoviruses,[1] APC[2] or RI,[3] associated with upper respiratory and other infections are related because of a common soluble antigen as demonstrated in the complement fixation test. With the neutralization test they have been separated into at least twelve types.[4] In order to learn more about the epidemiology of these viruses, the clinical syndromes associated with specific types, interrelations among types, and the response to vaccines, it is necessary to do neutralization tests.

The neutralization test originally described by Hilleman and Werner[3] and by Rowe and associates[5] consists of adding the serum and virus combination to HeLa cell tube cultures which have been grown out to a sheet of cells and washed free of human serum. Neutralizing antibody in the serum prevents cytopathogenesis by the virus. The amount of antibody is reflected in that dilution of the serum which prevents cytopathogenesis when the control tubes show advanced changes. This technique has been used successfully in this laboratory and elsewhere. However, the test is time consuming and difficulties have been encountered.[5,6] Some of the factors causing difficulty in the neutralization test are (a) the apparent variation in susceptibility of HeLa cell cultures to the stock adenoviruses, (b) the toxic effect of some human serums on HeLa cells, and (c) the determination of the time for reading the test. With respect to the latter problem the adenoviruses readily tend to break through the neutralizing effect of the antisera. Thus, the determination of the titer depends on an arbitrary time of reading. Enders[7] has discussed this problem of

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† Seymour Coman Fellow.

1. Enders, John F., Bell, J. A., Dingle, J. H., Francis, T. Jr., Hilleman, M. R., Huebner, R. J. and Payne, A. M.-M. 1956, "Adenoviruses": group name proposed for the new respiratory tract viruses. Science, **124**: 119–120.
2. Rowe, W. P., Huebner, R. J., Gilmore, L. K., Parrott, R. H. and Ward, T. G. 1953, Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. Proc. Soc. Exper. Biol. & Med. **84**: 570–573.
3. Hilleman, M. R. and Werner, J. H. 1954, Recovery of a new agent from patients with acute respiratory illness. Proc. Soc. Exper. Biol. & Med. **85**: 183–188.
4. Rowe, W. P., Hartley, J. W. and Huebner, R. J. 1956, Additional serotypes of the APC virus group. Proc. Soc. Exper. Biol. & Med. **91**: 260–262.
5. Rowe, W. P., Huebner, R. J., Hartley, J. W., Ward, T. G. and Parrott, R. H. 1955, Studies of the adenoidal-pharyngeal-conjunctival (APC) group of viruses. Am. J. Hyg. **61**: 197–218.
6. Jawetz, E., Kimura, S. J., Hanna, L., Coleman, V. R., Thygeson, P. and Nicholas, A. 1955. Studies on the etiology of epidemic keratoconjunctivitis. Am. J. Ophthalmol. **40**: 200–209.
7. Enders, J. F. 1954, The application of tissue cultures to the preparation and assay of viral antibodies. Ann. N. Y. Acad. Sc. **58**: 1072–1084.

"break-through" in reference particularly to poliomyelitis virus[8] and concluded that arbitrary end points in time should be used.

In the neutralization test described in this report, the serum, virus, and HeLa cells are added to culture tubes at the same time. The test is read within 24 to 48 hours. In this short period of time the adenovirus antigen causes typical cytopathogenesis while sufficient antibody prevents cytopathogenesis and allows normal growth of HeLa cells to take place. This neutralization test obviates some of the difficulties encountered in the test employed by others,[3,5,6] particularly that of "break-through." Considerable time also is saved by this simplified procedure and excellent repeatability has been demonstrated.

MATERIALS AND METHODS

HeLa cells and mediums.—The HeLa cell line used in this method was obtained originally from Dr. Jerome T. Syverton in 1953 and has been maintained in this laboratory since that time. This line now grows readily in culture medium to which either human or horse serum has been added. The stock cultures of HeLa cells were grown in Eagle's[9] medium containing 10% human serum. For the test, Eagle's medium with 10% horse serum‡ was used. All mediums contained 100 units of penicillin and 0.1 mg of streptomycin per ml.

Adenoviruses.—The RI-67 virus (type 4 adenovirus) was obtained from Dr. Maurice R. Hilleman. The remaining adenovirus prototypes 1 through 10 were supplied by Dr. Robert J. Huebner.

Preparation of virus antigens.—Stock HeLa cell cultures grown in 32 ounce prescription bottles were washed twice with maintenance medium (Eagle's medium with 5% horse serum) to remove the human serum. Maintenance medium was then added and the cultures inoculated with approximately 5 ml of stock prototype virus. After moderately advanced cytopathogenic changes (+++) had occurred (usually in 2 to 3 days) the fluids were harvested and replaced daily. The material from each day's harvest was frozen. Usually three or four daily yields of 25 ml per bottle were obtained. When several hundred ml were available the material was thawed, pooled and homogenized in a Waring blendor. The homogenized material was centifuged to remove large particles. The supernate was divided into 1 to 5 ml aliquots and refrozen. Stock antigen pools of types 3, 4 and 7 stored at −30 C have shown no loss of titer over a period of 1 year. Other adenovirus types have shown no variation after 6 months storage.

Rabbit antiserums.—Type specific antiserums were prepared by inoculating intravenously rabbits with 10 ml of a given type of adenovirus followed by the intraperitoneal inoculation of 10 ml of the same antigen one week later. The rabbits were then bled 1 month after the first injection.

HeLa cell suspensions for test.—Five- to seven-day-old stock HeLa cell cultures were washed twice with maintenance solution, after which 0.5% trypsin in Eagle's medium was added. After 30 minutes of incubation the resulting cell suspension was centrifuged and resuspended in Eagle's medium with 10% horse serum. The number of cells in the suspension was determined by direct count in a hemocytometer chamber. The cell suspension was then adjusted to give the desired concentration of approximately 100,000 cells in 0.7 ml.

Virus antigens for the test.—The amount of adenovirus of each type employed in the test was that amount without excess which produced ++++ cytopathogenesis in tubes of 100,000 HeLa cells when inoculated together with the cells under the conditions of the test. This amount was determined by making twofold dilutions of each stock virus to find that dilution causing about 50% cytopathogenesis. A 0.5 $\log_{10}$ stronger dilution of the stock was then employed in the test. The dilution of the stocks of the ten adenovirus types prepared for these studies which met the above definition varied from 1:2 to 1:30. Virus controls containing the amount used in the test and a 0.5 $\log_{10}$ dilution of that amount were included in each run.

Procedure of the test.—Serums to be tested were inactivated in a water bath at 57 C for 30 min-

‡ Obtained from Microbiological Associates, Inc., Washington, D. C.

8. Ledinko, N. and Melnick, J. L. 1953, Poliomyelitis virus in tissue culture. V. Reaction of virus and antibody; variables of the quantitative neutralization test. Am. J. Hyg. **58**: 223–247.

9. Eagle, H. 1955, The specific amino acid requirements of a human carcinoma cell (strain HeLa) in tissue culture. J. Exper. Med. **102**: 37–48.

utes. The following serum dilutions prepared in Eagle's medium were usually employed: 1:2, 1:4, 1:8, 1:32, 1:128, 1:512. 0.1 ml of serum dilution was used in each tube.* One tube per serum dilution was employed. Serum controls were tested only at the 1:2 serum dilution. Known specific rabbit antiserums were included as positive controls for each type of virus antigen. After the serum dilutions were added to the tubes 0.2 ml of the virus antigen was added with a Cornwall pipette. The tubes were shaken and the serum-virus mixture was allowed to stand at room temperature for 30 to 60 minutes. Seven tenths ml of the HeLa cell suspension containing approximately 100,000 cells was then added rapidly to each tube containing the serum-virus mixture with an automatic pipette (Brewer). The total volume in each tube was thus brought to 1 ml. The caps were then replaced and the tubes incubated at 37 C. The tubes were placed at about an 8° angle, slightly greater than the usual 5° in order to facilitate concentration of the cells.

Reading the test.—Each tube of the test was observed with the microscope under low power magnification after incubation for 24 to 48 hours. Readings at two days rather than at one day were preferred because the HeLa cells had formed more confluent sheets which allowed easier determination of the percentage of cytopathogenesis.† Each tube was graded as showing none or from + to ++++ cytopathogenesis. These symbols were interpreted to represent cytopathogenesis as follows: + = 1 to 25% of the cells; ++ = 26 to 50%; +++ = 51 to 75%; ++++ = 76 to 100%. Examples of none, ++ and ++++ cytopathogenesis at 48 hours under the conditions of the test are shown in figure 1.

The end point of the test was considered the greatest serum dilution allowing normal growth of the HeLa cells and no more than + cytopathogenesis. When fourfold serum dilutions were used and the first tube showing cytopathogenesis had ++ or +++ changes, the end point was taken as twofold less than the serum dilution in that tube. The titer was then expressed as the reciprocal of the original serum dilution.

RESULTS

Development of the neutralization procedure.—In evolving the neutralization procedure described above it was necessary to determine the optimal number of HeLa cells and the optimal strength of virus antigen. The interrelationship of these two factors is brought out in the experiment shown in table 1. In this test varying amounts of type 3 adenovirus were titrated with increasing numbers of HeLa cells. It can be seen that in the tubes containing the highest concentration of virus there was ++++ cytopathogenesis even in the tubes with 1,000,000 HeLa cells. As the amount of virus was diminished there was less cytopathogenesis. In those tubes inoculated with 1:31.6 dilution of type 3 adenovirus there was from ++ cytopathogenesis with 31,600 cells to no cytopathogenesis when mixed with 1,000,000 cells.

100,000 HeLa cell inoculums in 1 ml total volume were chosen for the routine test because this number provided enough cells so that the degree of cytopathogenesis could be determined both after 24 and 48 hours. Figure 2 shows

TABLE 1.—*The relationship of the number of HeLa cells to the degree of cytopathogenesis caused by different amounts of type 3 adenovirus when virus and cells were inoculated together and read 24 hours later.*

Adenovirus type 3 dilution	Number of HeLa cells inoculated per tube			
	31,600	100,000	316,000	1,000,000
1:1	++++*	++++	++++	++++
1:3.16	++++	++++	+++	+++
1:10	++++	++++	+++	++
1:31.6	+++	++	+	0

* Average reading of three tubes per dilution.

* For convenience when preparing a large number of serum samples to be tested the tubes containing the serum dilutions may be stored at 4 C for at least one week before performing the test without effecting the results. Pyrex screw cap test tubes (16×125 mm) and 64 place racks with spring holders were employed. The racks were obtained from the Drummond Scientific Company, Philadelphia, Pennsylvania.

† Although the tubes were somewhat more difficult to read after 24 hours it was frequently helpful to be able to divide the reading of a large run between the first and second day. No difference in titer was found between one and two day readings presumably because the multiplication of normal HeLa cells balanced the increased viral cytopathogenesis. Positive serum and virus controls were always read on both days.

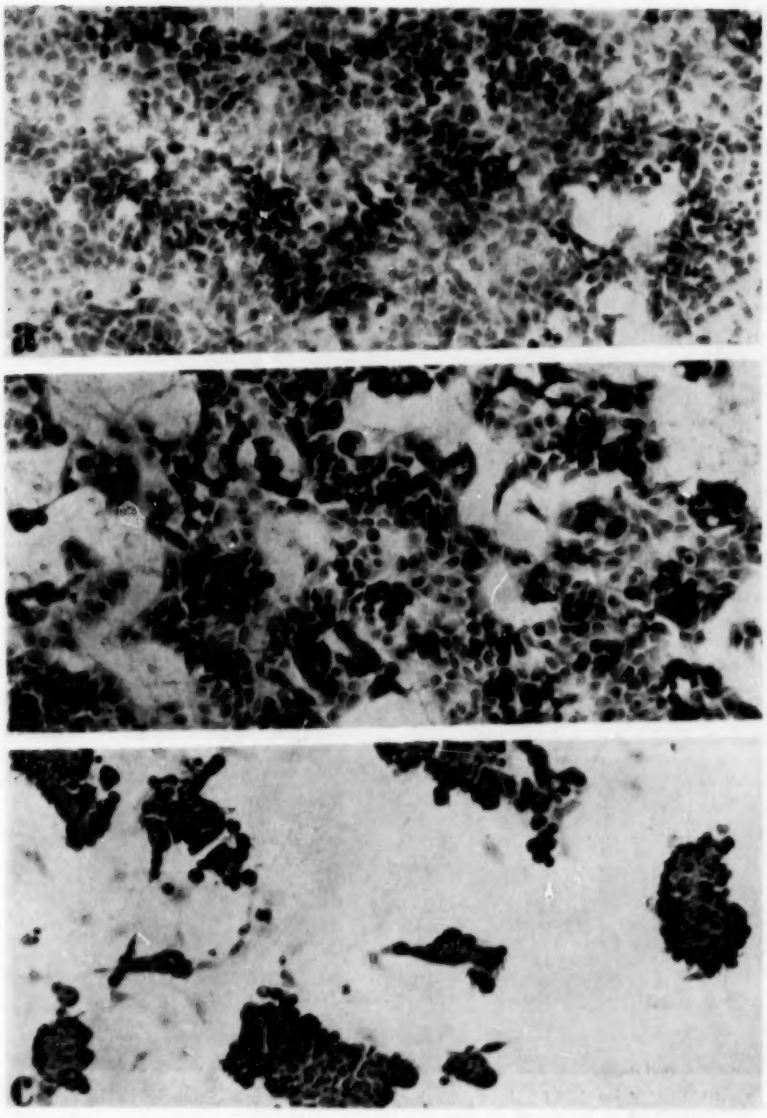

FIG. 1.—Photomicrographs of examples of cell and virus controls in the neutralization test described in this paper. Each example is a 2-day growth of an original inoculum of 100,000 HeLa cells in Eagle's medium containing 10% horse serum. Type 3 adenovirus was added along with the HeLa cells as follows:

a. No virus added. The HeLa cells have grown into a sheet and show no cytopathogenesis.

b. Virus added in the amount of 0.5 $\log_{10}$ dilution of that used in test (1:31.6 of stock). The HeLa cells show + + cytopathogenesis.

c. Virus added in the amount of that used in test (1:10 dilution of stock). The HeLa cells show + + + + cytopathogenesis.

Tissue fixed with 10% formalin in alcohol (95%) and stained lightly with hematoxylin and eosin. ×110.

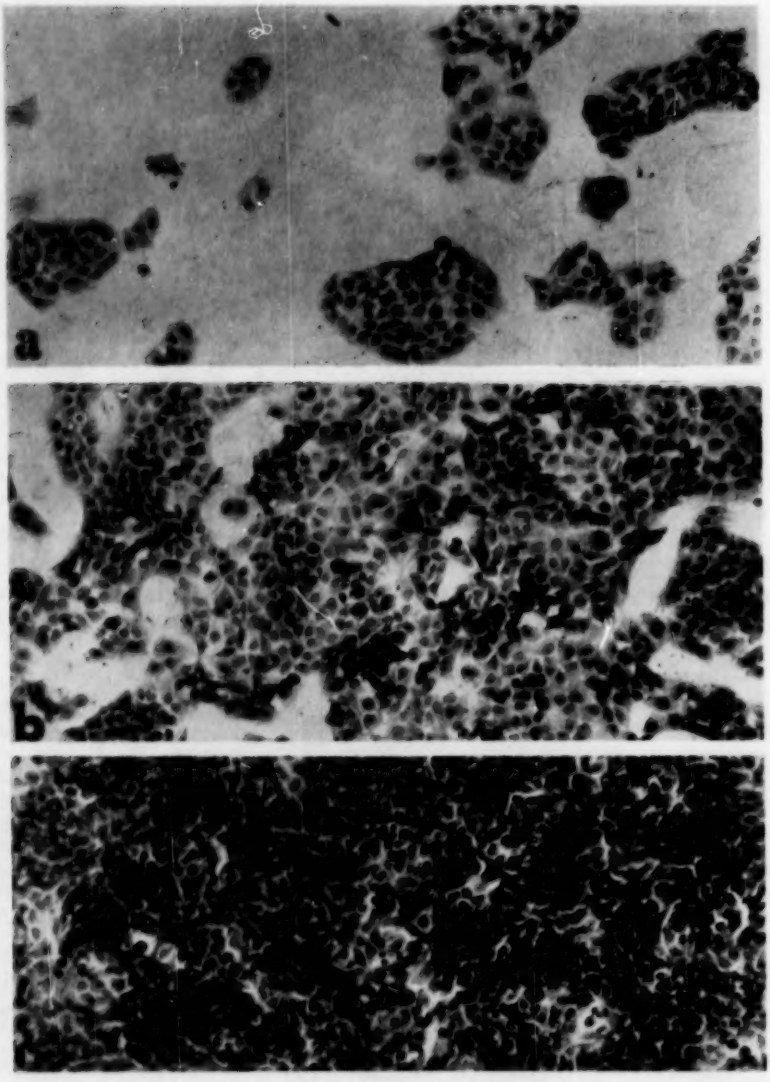

FIG. 2.—Photomicrographs of 2 days growth of the HeLa cell line employed in the neutralization test.

a. Inoculum 31,600 cells in 1 ml Eagle's medium containing 10% horse serum.
b. 100,000 cell inoculum.
c. 316,000 cell inoculum.

Tissue fixed with 10% formalin in alcohol (95%) and stained lightly with hematoxylin and eosin. ×110.

TABLE 2.—*Titration of type 3 adenovirus against type specific rabbit antiserums in the presence of 100,000 HeLa cells per tube. The serum, virus and cells were added to the tubes at the same time and cytopathogenesis read at 24 hours.*

Adenovirus type 3 dilution	Serum dilution						Virus control
	1:8	1:32	1:128	1:512	1:2048	1:8192	
1:1	+	++++	++++	++++			++++
1:3.16	0	0	+++	++++	++++		++++
1:10			0	0	+++	++++	++++
1:31.6			0	0	0	++	++
1:100			0	0	0	0	0

the growth after 2 days with three different sized HeLa cell inoculums. The thin HeLa cell sheet resulting from the 100,000 cell inoculum can be seen in figure 2b and contrasted with scattered islands of cells seen in figure 2a with a 31,600 cell inoculum and the thick layer of cells in figure 2c following a 316,000 cell inoculum.

Various stock adenoviruses were titered in the presence of rabbit type specific antiserums and known positive human serums in order to determine the optimal amount of virus antigen for the test. Table 2 presents an example of such a box titration of adenovirus type 3 against its type specific rabbit antiserums. It can be seen in this table that the 1:10 dilution of the stock virus was the highest dilution still giving ++++ cytopathogenesis in the control tubes. The higher concentrations of virus antigen (1:3.16 and 1:1) also produced ++++ cytopathogenesis in the control tubes.* However, these dilutions of antigen reduced the neutralizing titer 16 and 128-fold from the titer obtained in the 1:10 dilution of virus. A similar relationship of excess viral antigen reducing serum neutralizing titer was shown for the other adenovirus types. Therefore, in order to provide a sensitive test, that dilution of stock virus without excess virus causing ++++ cytopathogenesis under conditions of the test was employed as antigen.

It can also be seen in table 2 that the 0.5 $\log_{10}$ dilution (1:31.6) of the optimal antigen strength caused ++ cytopathogenesis in the control tubes. This relationship between the optimal concentration of virus antigen and its 0.5 $\log_{10}$ dilution was demonstrated for each adenovirus type and provides the basis for titration of stock virus antigen detailed in the section on Materials and Methods.

In table 2 the serum neutralizing titer obtained with the 1:10 dilution of virus antigen could well be taken as 512, the highest dilution completely preventing cytopathogenesis. However, the next fourfold serum dilution tube (1:2048) showed some evidence of neutralization as indicated by a +++ cytopathogenesis reading. It was decided to interpolate the end point in the case of fourfold serum dilutions to that titer twofold less than that tube showing partial (++ to +++) cytopathogenesis, in this case to 1024. The validity of such interpolation was confirmed by running twofold dilutions with serum samples giving a variety of positive titers. Thus, the serum titers assigned to the results obtained in table 2 were as follows: with the 1:1 dilution of virus, 8; with the 1:3.16 dilution of virus, 64;

* Even when virus was present in considerable excess of the amount necessary to produce ++++ cytopathogenesis of HeLa cells some infected cells still attached to the glass surface and formed grape-like clusters with nuclear abnormalities typical of cytopathogenesis due to adenovirus. The acid production typical of adenovirus growth also occurred when excess virus was present.

TABLE 3.—*Results of repeated neutralization tests with rabbit type specific antis-rums using two different techniques.*

Adenovirus type	Number of runs with the following neutralizing titers											
	64		128		256		512		1024		2048	
	A	B*	A	B	A	B	A	B	A	B	A	B
1	2	1		2								
2					2		1	3				
3					1		2	8		1		1
4							2	6				
5					2	2	1	1				
6					1			3	2			
7							3	7	1	1		1
8		1		2								
9		3										
10		1		1		1						

* A = neutralization procedure after Hilleman and Werner;[3]
B = neutralization procedure described in this paper.

and with the 1:10 dilution of virus, 1024.

Rabbit antiserums.—Rabbit type specific antiserums against adenovirus types 1 through 7 had been tested at least twice with the neutralization technique after Hilleman and Werner.[3] These same serums plus types 8, 9 and 10 have now been tested several times with the technique described in this paper. Table 3 shows that the titers obtained with the two techniques fall in the same general range. Types 2 through 7 tended to have titers of 512 while types 1, 8, 9 and 10 were 64 or 128. Types 3, 4 and 7 have been run six to ten times with the newly described technique with a range of titers no greater than fourfold (one tube).

Human serums from adenovirus infection.—Paired serum samples were available from 53 persons suffering acute respiratory illness and having an adenovirus isolated from their upper respiratory tract.[10] Neutralization titers had

TABLE 4.—*Studies of the repeatability of results using the neutralization technique after Hilleman and Werner.[3] Results tabulated include 395 repeat determinations of neutralizing antibody titers with types 3, 4 and 7 adenovirus antigens in both acute and convalescent serums from 53 persons suffering an acute respiratory infection and having an adenovirus isolated from their upper respiratory tract.*

Titer on repeated run	Serum neutralizing titer on first run							
	<8	8	16	32	64	128	256	512
<8	185	31	7	4	2			
8	16	9	7	4	2			
16	6	16	8	1	3	4		
32	5	8	5	6	6	7	3	
64			1	1	7			
128		1			5	3	1	2
256			2	2	5	8	1	
512				1	2	3	5	

TABLE 5.—*Studies of the repeatability of results using the neutralization technique described in this paper. Results tabulated include 531 repeat determinations of neutralizing antibody titers with adenovirus types 1 through 10 in both acute and convalescent serums from 53 persons suffering an acute respiratory infection and having an adenovirus isolated from their upper respiratory tract.*

Titer on repeated run	Serum neutralizing titer on first run										
	<2	2	4	8	16	32	64	128	256	512	1024
<2	201	23	4								
2	21	8	4	1							
4	6	7	2	10							
8		3	5	23	9	7					
16			1	4	4	9					
32				4	10	47	9	7	1		
64						15	3	6	2		
128						4	13	27	6	3	
256							1	5		2	2
512								1	2	5	3
1024										1	

been determined against types 3, 4 and 7 adenovirus antigens with the original technique of Hilleman and Werner.[8] At least two separate determinations were available for each pair against the three antigen types and three or four separate neutralization tests had been run on some of the serum pairs. The results obtained with a total of 395 repeat determinations compared to the original run are shown in table 4.

These same paired serums have now been tested with types 1 through 10 adenovirus antigens utilizing the technique described in this paper. The acute and convalescent serums have all been run against the ten antigens once[11] and a total of 531 repeat tests have been performed. A comparison of the repeat result with the titer obtained when first tested is presented in table 5.

Inspection of tables 4 and 5 reveals that considerably closer agreement between the original and the repeat neutralization test was obtained with the technique described in this paper. With this technique (table 5) only one repeat

10. Woolridge, R. L., Grayston, J. T., Whiteside, J. E., Loosli, C. G., Friedman, M. and Pierce, W. E. 1956, Studies on acute respiratory illness in Naval recruits with emphasis on the adenoviruses (APC-RI). J. Infect. Dis. **99**: 182–188.

11. Grayston, J. T., Loosli, C. G., Johnston, P. B., Smith, M. E. and Woolridge, R. L. 1956, Neutralizing antibody response to adenovirus infection. J. Infect. Dis. **99**: 199–206.

test failed to agree with the original titer within fourfold. Since fourfold dilutions were employed this agreement is within a one tube variation. On the other hand, with the original technique of Hilleman and Werner[3] (table 4) 28 of the repeat tests varied from the first titer by 8- or 16-fold. The failures to repeat the original titer were not particularly associated with any one of the three antigens. It is possible that even more variation is hidden by the fact that the 1:8 serum dilution was the lowest dilution employed with this technique. Table 4 shows that the 28 failures to repeat within fourfold represent 25% (28/110) of those repeats in which a greater than fourfold variation was possible (one titer of 32 or greater). In addition to the poorer repeatability with the original technique[3] about one in five of the runs had to be discarded because the virus controls did not show the proper amount of cytopathogenesis within the 2 to 4 days after inoculation when the test was to be read. In the studies on repeatability described above only one tube per serum dilution was employed in the newer technique. Thus, additional time was saved compared to the two tubes per dilution necessary with the original technique.[3]

The average or most often repeated neutralizing antibody titers obtained with the two test procedures on the 53 paired human serums are compared in table 6. Since three antigens (types 3, 4 and 7) were run with both procedures, there was a total of 318 comparisons. As can be seen in table 6, 307 of the titers agreed within fourfold. There were nine titers which varied by 8-fold and two

TABLE 6.—*Comparison of neutralization titers obtained by two different test procedures against types 3, 4 and 7 adenovirus antigens on the same 53 paired serums from patients suffering upper respiratory infections from whom an adenovirus was isolated.*

Titer with technique B*	Titer with technique A							
	<8	8	16	32	64	128	256	512
<2	136	2						
2	26	3	1					
4	11	3						
8	5	13	4	2	3	1		
16	2	2	7	5		2		
32	1	1	5	13	10	7		
64			2	2	6	4	3	
128		1		3	2	10	2	
256						1	1	
512						3	5	1

* Technique A after Hilleman and Werner;[3] technique B described in this paper.

varied by 16-fold. A rise in titer of fourfold between the acute and convalescent serum against one of the antigens was found with one procedure but not the other, four times with the newer technique described in this paper and once with the original technique.[3]

The homotypic and heterotypic neutralizing antibody response to ten adenovirus antigens found in these 53 human serum samples is reported in a companion paper.[11] In addition to the rabbit antiserum and the human serum results described above, the newer technique has been employed to determine adenovirus neutralizing antibodies in some 500 human serums from different sources. Throughout this experience the technique has given rapid and clear-cut results. No test run has had to be discarded. Only three human serums have shown "toxic" effects and only once did this prevent determining cytopathogenesis at the 1:2 level.

DISCUSSION

In an effort to simplify the neutralization procedure with adenovirus a "line" of HeLa cells that would grow in tubes without human serum was sought so as to avoid the need for washing the tubes prior to use. When one line carried in the laboratory was found to grow vigorously in a 10% horse serum medium further simplification was attempted by earlier addition of the serum and virus. It was found that not only was the procedure simplified but that more clear-cut and more easily repeatable results were obtained when the serum and virus were added to tubes at the same time as the HeLa cells. The HeLa cell indicator system is not only more easily quantitated under these conditions but appears to be more sensitive prior to development of cell sheets on the glass surface. A HeLa cell line that grows vigorously in the absence of human serum is a prerequisite for the successful employment of the neutralization procedure described in this paper. Another HeLa cell line carried in this laboratory* works poorly in the described test procedure due to irregular growth in the horse serum medium.

The importance of quantitation of the HeLa cell culture has been brought out in these studies. One of the causes of the apparent variation in the amount of viral antigen needed to produce the desired amount of cytopathogenesis within 2 to 4 days in the originally described neutralization techniques[3,5] may have been different numbers of cells in the tubes at the time they were used.

The difficult problem of how long a serum should be expected to "neutralize" the virus in a tissue culture system is avoided with the procedure described. In the originally described neutralization techniques, break-through the neutralizing effect of antiserums occurs very readily with adenovirus. When adenovirus is added to the HeLa cells in suspension there appears to be a rather constant relationship between infected and uninfected cells for at least 48 hours. This is supported by the finding that readings of percentage cytopathogenesis made at 24 and 48 hours are usually the same. Tubes showing complete neutralization at 24 hours never have shown cytopathogenesis at 48 hours.

That this neutralization procedure read in 1 or 2 days measures the same neutralizing antibodies found with the more conventional procedures is supported by the evidence presented that serum titers of both rabbit antiserums and postinfection human serums were generally similar with both techniques.

* Obtained in 1954 from Microbiological Associates, Inc., Washington, D.C.

Further simplification of the neutralization test for adenovirus along the lines of the color test neutralization procedure for poliomyelitis[12] would be desirable. Since acid is produced in the presence of excess adenovirus the same reaction used for poliomyelitis is not feasible. However, the fact that adenovirus infected HeLa cell cultures produce more acid than noninfected cultures may provide the basis for a color reaction neutralization test. The development of such a test is under study in this laboratory.

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SUMMARY

A simplified neutralization test for adenoviruses has been described. HeLa cells that will grow vigorously in medium containing horse serum serve as the indicator system and are added in suspension at the same time as the serum and virus. The test is read by microscopic observation at 1 or 2 days. During this time the viral antigen causes ++++ cytopathogenesis while serum containing sufficient neutralizing antibody prevents cytopathogenesis and allows normal growth of the HeLa cells. Evidence is presented to show that the test procedure gives consistent, repeatable results and that the titers obtained are similar to those found with the conventional method.

NEUTRALIZING AND COMPLEMENT FIXING ANTIBODY RESPONSE TO ADENOVIRUS INFECTION

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The appearance of neutralizing and complement fixing antibodies was reported along with the original isolations of adenoviruses[1] from military[2] and civilian[3] persons suffering from respiratory illnesses. Whereas the complement fixing antigen is a group antigen, the neutralization test has allowed the establishment of at least twelve separate types of adenovirus.[4] Different clinical and epidemiological patterns have been described for some of these types.[4–8] Although rabbit type specific antiserums do not show significant cross reactions with other adenovirus types,[4] some heterotypic rises have been noted following human adenovirus infection.[2,9] The studies reported in this paper are mainly concerned with the homotypic and heterotypic neutralizing antibody response against ten adenovirus types in paired serums from 53 persons suffering acute upper respiratory illnesses and from each of whom an adenovirus, type 3, 4 or 7, was isolated.

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MATERIALS AND METHODS

Fifty of the paired serums came from Naval recruits, at the United States Naval Training Center, Great Lakes, Illinois, who were involved in a study of acute respiratory illnesses.[10] The remaining three paired serums came from male patients of the University of Chicago Clinics. The paired serums were usually 3 weeks apart but varied from 2 to 4 weeks. All serums were stored at −30 C. An adenovirus was isolated and typed from each person whose paired serums is included in these studies. The source of the virus was either a nasal or a throat washing. All viruses were isolated in HeLa cell cultures. More detail on the methods employed in study of these patients is presented in a companion paper.[10]

The prototype adenoviruses, types 1 through 10, employed as antigens in this study were obtained from Dr. Maurice R. Hilleman (type 4) and Dr. Robert J. Huebner (remaining types). The antigens were grown in HeLa cell cultures employing Eagle's medium with 5% horse serum as maintenance medium. Type specific antiserums were prepared by inoculating intravenously rabbits with 10 ml of a given type of adenovirus followed by the intraperitoneal inoculation of 10 ml of the same antigen 1 week later. The rabbits were then bled 1 month after the first injection.

In the new simplified neutralization test[11] employed in this study, the serum, virus and HeLa cells are added to culture tubes at the same time and the test is read within 24 to 48 hours. In this short period of time adenovirus antigen causes typical cytopathogenesis while sufficient antibody prevents cytopathogenesis and allows normal growth of HeLa cells to take place. The complement fixing antigen used was type 4 adenovirus. It was prepared in the same manner as the neutralizing antigen and then inactivated at 56 C for 1 hour.[10] The technique of the complement fixation test was the same as previously described.[12] In the complement fixation and neutralization tests an antibody rise of fourfold or greater between the acute and convalescent serums was interpreted to be significant. Serum dilutions were tested from 1:2 to 1:512 in the neutralization test and from 1:4 to 1:512 in the complement fixation test. The antibody titers reported are reciprocals of the original serum dilution. The production of antigen and antiserums and the neutralization test procedure employed are described in greater detail in the preceding paper.[11]

TABLE 1.—*Neutralizing antibody response to the isolated adenovirus prototype and complement fixation antibody response to adenovirus antigen in the convalescent serum of 53 persons suffering acute respiratory illness and having an adenovirus isolated from their upper respiratory tract.*

Adenovirus type isolated	Number of persons	Number of convalescent serums with the following antibody titer*						
		8	16	32	64	128	256	512
Neutralizing antibody								
3	5			1	2	2		
4	25	4	2	7	6	4		2
7	23	1	3	8	1	9		1
Total	53	5	5	16	9	15	0	3
Complement fixing antibody								
3	5		1	1	2	1		
4	25		2	6	7	5	4	1
7	23			5	4	5	6	3
Total	53	0	3	12	13	11	10	4

* Titer is the reciprocal of the original serum dilution.

RESULTS

All of the 53 individuals whose serums were used in this study had an acute febrile respiratory illness at the time acute serums and washings of the upper respiratory tract were taken. Three types of adenovirus were isolated from these samples. The viruses included 5 type 3, 25 type 4, and 23 type 7. All paired serums had significant complement fixing antibody titer rises. All but two individuals (one with type 4 and one with type 7) had a significant neutralizing antibody titer rise in the convalescent serums to the prototype of the virus isolated. The range of neutralizing antibody titer in the convalescent serums to the prototype virus is shown in table 1. It can be seen in this table that

10. Woolridge, R. L., Grayston, J. T., Whiteside, J. E., Loosli, C. G., Friedman, M. and Pierce, W. E. 1956, Studies on acute respiratory illness in Naval recruits with emphasis on the adenoviruses (APC-RI). J. Infect. Dis. **99**: 182–187.
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of the 53 convalescent serums, 43 had titers of 32 to 512, while 10 had titers of 8 and 16. There was low titer neutralizing antibody in 5 of the acute serums against the prototype virus isolated. The two pairs failing to show rises both had acute neutralizing antibody (at 4 and 16). The other three with acute antibody (at 2, 4 and 8) had greater than fourfold rises. Also in table 1 the complement fixing antibody titer in the 53 convalescent serums is shown. Three show a titer of 16 with the rest distributed from 32 to 512. Fourteen of the pairs had complement fixing antibody in the acute serum ranging from 4 (the lowest dilution tested) to 32. Complement fixing antibodies were not demonstrated in any of the 5 acute serums that had neutralizing antibody against the isolated adenovirus type.

Rabbit type specific antiserums against adenovirus types 1 through 10 were tested for cross reactions in the neutralization test. These data are presented in table 2. It can be seen that neutralizing antibody titers were from 64 to 512 against the homologous antigen. There were only occasional low titered reactions against the other nine antigens except with antiserums prepared against adenovirus prototype 9. Serum from the rabbit immunized with type 9 showed a titer of 32 with antigen type 8 compared to a homotypic response of 64.† Antiserums prepared with prototype 8 failed to react with type 9 virus antigen. Otherwise, only a few titers of 2 and 4 were found with a cross reaction between types 3 and 7 at the titer of 4 being the only reciprocal relation.

In addition to the neutralizing antibody rises in the human paired serums against the virus type recovered some rises were also observed against the other ten antigens employed. Table 3 presents the results obtained with neutralization and complement fixation tests on the 53 paired serums. This detailed table is presented to enable the reader to see the actual titers obtained, their range and interrelations. Cursory inspection of this table shows the frequency of such heterotypic rises, both in the presence and in the absence of antibody in the acute serum. The general absence or low titer neutralizing antibody with types 8, 9 and 10 is in contrast to the frequent high titer antibody observed with the other types. The subsequent tables to be presented are calculated from these basic data and specific paired serums will be referred to by patient number in the following discussion.

The heterotypic rises occurring with

TABLE 2.—*Cross neutralizing antibody test with type specific rabbit antiserums against adenovirus types 1 through 10.*

Antiserum type	Neutralizing antibody titer with adenovirus types									
	1	2	3	4	5	6	7	8	9	10
1	128	0*	0	0	0	0	0	0	0	0
2	0	512	2	0	2	0	0	0	0	0
3	0	0	512	0	0	0	4	0	0	0
4	0	0	0	512	0	0	0	0	0	0
5	0	0	0	0	512	0	0	0	0	0
6	0	0	0	0	4	512	0	0	0	0
7	0	0	4	0	0	0	512	0	0	0
8	0	0	0	0	0	0	0	128	0	0
9	0	0	0	0	0	4	0	32†	64	0
10	0	0	0	0	0	0	0	0	2	128
Anti Hela control	0	0	0	0	0	0	0	0	0	0

* 0 = <2.

† Antiserums from immunization of a second and third rabbit with type 9 adenovirus failed to cause significant cross reactions with type 8 antigen. In addition, a higher homologous titer of 1024 was elicited.

TABLE 3.—*Neutralizing and complement fixing antibody titers with adenovirus antigens in paired serums from 53 persons suffering acute respiratory illness and having an adenovirus isolated from their upper respiratory tract.*

Patient no.	Adenovirus type isolated	Complement fixation titer	Neutralization titer of acute over convalescent serum									
			Adenovirus type:									
			1	2	3	4	5	6	7	8	9	10
2003	3	0/64	0/0	32/32	0/64	0/0	0/0	0/0	0/0	0/0	0/0	0/0
2006	3	0/64	8/8	0/0	0/128	0/2	32/32	0/0	0/0	0/0	0/0	0/0
2016	3	0/16	2/4	32/128	4/64	0/0	4/8	4/4	0/0	2/2	0/0	2/4
2238	3	0/128	16/8	16/32	0/128	0/16	32/32	0/2	0/2	0/4	0/2	0/8
W369	3	0/32	0/0	0/0	0/32	0/0	128/128	0/0	0/0	0/0	0/0	0/0
2312	4	0/256	8/16	0/8	0/16	0/512	16/32	0/4	0/0	0/0	0/0	0/0
2330	4	16/64	16/16	8/32	32/128	0/32	0/2	8/4	32/32	0/0	0/0	0/0
2348	4	0/64	16/32	0/8	8/32	0/32	128/256	0/16	0/0	0/2	0/0	0/4
2353	4	0/64	8/32	8/32	32/64	0/8	0/0	8/32	8/8	0/0	0/0	0/0
2404	4	8/256	16/32	2/2	32/32	0/128	64/64	4/4	0/0	0/0	0/0	2/4
2405	4	0/128	32/32	2/8	8/128	0/32	0/8	0/8	0/2	0/2	0/4	0/2
2408	4	0/128	2/8	32/32	32/32	0/128	0/0	8/8	0/0	0/0	0/0	0/0
2410	4	0/32	0/0	0/0	8/4	0/64	0/0	0/0	0/0	0/0	0/0	0/0
2412	4	0/128	0/0	0/0	64/128	0/16	0/0	0/0	0/8	0/0	0/0	0/8
2426	4	16/256	0/0	0/0	2/8	0/64	0/0	0/0	8/8	0/0	0/2	8/4
2427	4	0/64	0/0	32/32	32/32	0/32	0/0	0/2	0/4	0/2	0/0	0/2
2428	4	0/32	16/8	0/0	0/0	0/64	0/0	0/8	0/0	0/0	0/0	0/0
2444	4	0/32	0/0	0/0	0/0	0/32	128/128	0/0	0/0	0/0	0/0	4/8
2445	4	0/512	16/4	0/32	4/32	0/8	0/0	0/8	2/4	0/0	0/0	0/2
2455	4	0/64	0/4	16/128	32/256	0/512	32/32	0/8	0/128	0/4	0/2	0/4
2458	4	0/32	0/0	128/128	32/32	0/64	4/4	0/0	0/2	0/0	0/0	0/0
2459	4	0/128	0/4	32/128	4/8	0/64	32/128	0/16	0/32	0/0	0/4	0/8
2464	4	0/32	32/32	32/32	16/32	4/8	128/128	128/128	4/8	0/0	0/0	2/4
2465	4	0/16	0/0	0/0	0/0	0/64	0/0	0/4	0/0	0/0	0/0	0/0
2466	4	0/256	0/0	64/64	0/0	0/32	128/128	0/4	16/8	0/0	0/0	0/0
2467	4	0/128	0/0	0/0	32/64	0/128	64/64	0/0	0/0	0/0	0/0	0/0
2468	4	0/16	8/8	8/8	0/2	0/8	8/32	8/32	0/2	0/0	0/0	0/2
2478	4	0/64	32/32	32/32	16/8	0/32	0/4	32/32	0/2	0/0	0/0	0/2
W277	4	0/32	0/2	64/128	0/8	0/16	0/0	0/8	0/4	0/2	0/0	0/0
S1018	4	0/64	4/16	31/128	32/128	8/128	32/64	8/128	0/0	0/0	0/4	0/0
2185	7	4/32	0/8	128/128	0/0	0/0	0/0	0/0	0/32	4/4	0/0	0/2
2187	7	0/32	0/0	0/0	16/512	0/0	0/0	0/0	0/128	0/4	0/0	0/0
2196	7	8/64	4/4	128/128	2/16	4/16	0/0	8/8	0/32	0/2	0/4	0/0
2223	7	4/512	0/8	32/32	0/64	0/8	8/128	0/16	0/512	0/0	0/4	0/0
2224	7	0/64	0/0	128/128	0/0	2/2	8/256	0/2	0/128	0/0	0/0	0/0
2254	7	0/256	4/4	32/32	8/512	0/0	8/32	8/8	0/32	0/0	0/0	0/0
2258	7	32/256	2/0	128/128	0/2	0/0	0/0	0/2	0/64	0/4	0/0	0/0
2264	7	0/32	64/64	8/2	4/4	0/0	32/32	4/2	0/128	0/0	0/2	2/2
2266	7	16/128	32/64	32/32	2/2	0/2	0/0	8/4	0/32	4/4	0/0	0/0
2270	7	0/32	16/16	64/64	16/512	0/0	0/0	8/8	0/128	0/0	0/0	0/0
2280	7	0/128	0/0	64/64	2/0	0/0	16/32	2/2	0/32	0/0	0/0	0/0
2283	7	0/256	4/8	128/128	4/4	0/0	0/0	8/8	0/128	0/0	0/0	0/0
2291	7	0/32	8/32	32/32	32/32	0/32	32/32	32/32	16/16	4/4	0/0	0/0
2300	7	0/128	16/16	32/32	2/2	0/32	0/2	8/4	0/32	0/8	0/2	0/0
2308	7	8/256	0/0	0/0	0/2	0/2	128/128	0/0	0/16	0/0	0/0	0/0
2309	7	32/128	16/32	512/512	0/16	4/16	0/2	2/2	0/16	0/8	0/0	0/4
2311	7	8/64	32/32	8/8	8/32	0/64	2/2	8/8	0/8	0/8	0/4	0/2
2314	7	0/256	2/2	8/8	8/512	0/0	0/0	8/8	4/128	0/8	0/0	0/0
2315	7	8/64	0/0	32/32	0/8	2/16	0/0	0/0	0/32	2/8	0/4	0/4
2316	7	0/512	32/16	32/32	8/512	8/8	32/8	8/8	0/128	0/0	0/0	0/0
2318	7	8/512	4/16	32/32	16/512	0/128	128/512	2/16	0/128	0/16	0/0	0/0
2324	7	16/128	16/16	2/2	2/32	0/8	16/32	8/8	0/128	0/0	0/0	0/0
2373	7	0/256	2/4	128/128	2/2	0/8	0/0	0/0	0/32	0/0	0/4	0/0

antigens other than the adenovirus type isolated are summarized by antigen type and by isolated virus type in table 4. It can be seen that the total number of heterotypic rises in serum pairs from patients yielding types 4 and 7 virus were nearly identical (23 and 22%). Only 9% heterotypic rises were found in the few type 3 infections. Heterotypic rises occurred with each of the ten antigen types in at least 13% of the patients irrespective of virus type isolated. Such rises were seen most frequently with types 3 and 4 as antigen (44 and 39% respectively). Twenty-eight percent of the serum pairs had rises with type 6 virus and 19% with type 2. Heterotypic rises against these two types were associated mostly with type 4 virus isolations. Nineteen percent of the serum pairs showed rises against type 8, mostly in persons infected with type 7 virus.

The distribution of neutralizing antibody in the acute serums of the 53 patients is summarized in table 5. At the

TABLE 4.—*Heterotypic neutralizing antibody rises for adenovirus types 1 through 10 in paired serum samples from 53 persons suffering acute respiratory illness and having an adenovirus isolated from their upper respiratory tract.*

Virus type isolated	Number of persons	Number of fourfold or greater neutralizing antibody rises to adenovirus type										Total rises	Percent
		1	2	3	4	5	6	7	8	9	10		
3	5	0	1		1	0	0	0	1	0	1	4/45	9
4	25	5	9	9		4	13	4	1	3	4	52/225	23
7	23	3	0	12	10	4	2		8	5	2	46/207	22
Total rises in percent		15	19	44	39	15	28	13	19	15	13		

level of 1:2 serum dilution, antibody was present against types 1, 2, 3, 5 and 6 in from 45 to 74% of the men. Considerably less acute serum antibody was found against types 4 and 7 (18 and 20%), even though the persons infected with these types are excluded. No acute serum showed antibodies against type 9, while 6 (11%) showed type 10 and 5 (9%) showed type 8 antibodies. High titer neutralizing antibodies (16 and above) in the acute serums were associated almost exclusively with adenovirus types 1, 2, 3 and 5. They were not found at all in these patients with antigen types 4, 8, 9 and 10. Complement fixing antibodies were found in one quarter of the acute serum samples. They tended to be more frequent in persons from whom type 7 virus was isolated. No type of acute neutralizing antibodies was predominantly associated with any of the three infecting adenovirus types although serums of persons from whom type 7 was isolated did have more frequent acute antibody to most of the antigens.

The frequency of heterotypic rises observed in each serum pair varied widely. These number are presented in table 6. It can be seen that of the 53 serums examined 14 pairs had no heterotypic rise, while the other 39 had a heterotypic rise to at least 1 of the 9 antigens. Thirteen had a heterotypic

TABLE 6.—*Number of heterotypic neutralizing antibody responses against adenovirus types 1 through 10 in paired serums from 53 patients suffering acute respiratory illness and having an adenovirus isolated from their upper respiratory tract.*

Virus type isolated	Number of persons	Number of serum pairs showing the indicated number of heterotypic rises against the 9 nonisolated adenovirus types							
		0	1	2	3	4	5	6	7
3	5	3	1		1				
4	25	6	7	4	3	1	2		2
7	23	5	5	7	1	2	1	2	
Total	53	14	13	11	5	3	3	2	2

TABLE 5.—*The number of serums with complement fixing and neutralizing antibodies against adenovirus antigens in the acute serum sample from 53 persons suffering acute respiratory illness and having an adenovirus isolated from their upper respiratory tract.*

Adenovirus type isolated	Number of persons	Complement fixation	Adenovirus antigen type									
			1	2	3	4	5	6	7	8	9	10
			Number with antibody at 1:2 or greater serum dilution*									
3	5	0	3	3	†	0	4	1	0	1	0	1
4	25	3	12	15	18		12	8	6	0	0	4
7	23	11	16	21	16	5	11	15		4	0	1
Total in percent		26	60	74	71	18	51	45	20	9	0	11
			Number with antibody at 1:16 or greater serum dilution									
3	5	0	1	3		0	3	0	0	0	0	0
4	25	2	8	10	12		10	2	2	0	0	0
7	23	4	8	17	4	0	7	1		0	0	0
Total in percent		11	32	57	33	0	38	6	7	0	0	0

* Complement fixation 1:4 or greater.
† Antibodies against the infecting type excluded.

rise to 1 antigen, 11 to 2 antigens, 5 to 3 antigens and 2 or 3 of the serum pairs had 4, 5, 6 and 7 heterotypic rises. The reactions of the four pairs showing 6 and 7 heterotypic rises are shown in table 3 under the patient numbers 2455, 2459, 2223, and 2318.

As also can be seen in table 3 the height of the titer of the heterotypic responses varied for the different types. Some of this variation depended on the presence of acute neutralizing antibody. Antibody titers to types 8, 9 and 10 in the convalescent serums only once exceeded 8 (patient no. 2316). On the other hand, heterotypic neutralizing titers of 128 were seen in the absence of acute antibody with types 3, 4 and 7 as antigen. Heterotypic responses of greater titer than 32 were not seen with types 1, 2, 5 and 6 as antigen in the absence of acute antibody. Almost all of the heterotypic responses seen with antigen types 1, 2, 5, 8, 9 and 10 were 4- or 8-fold rises. With types 3, 4 and 7, 16-fold or greater neutralizing antibody rises occurred in about one-half of those showing heterotypic rises.

In 8 of the 51 serum pairs showing a homotypic rise there was a heterotypic neutralizing titer rise as great (table 3, patient no. S1018, 2300, 2309, 2318) or greater (patient no. 2445, 2254, 2311, 2314) than the homotypic rise. Six of these eight pairs were from persons who had type 7 adenovirus isolated from their nasal washings. In addition, the serum pair failing to show a homotypic neutralizing titer rise against type 7 (patient no 2291) did show a rise from 0 to 32 against type 4 antigen and from 8 to 32 with type 1. In addition acute serum high titer neutralizing antibody was present in patient number 2291 against antigen types 2, 3, 5 and 6. The pair failing to show a homotypic rise against type 4 (patient no. 2464) had neutralizing antibodies in the acute serum against all types except 8 and 9 yet failed to show a neutralizing rise against any type. However, a significant complement fixation antibody titer rise was demonstrated with this pair. In spite of the acute neutralizing antibodies (some of high titer) present in this patient (no. 2464) to 8 of the 10 adenovirus antigens no complement fixing antibodies were found in the acute serum. This is a good example of the general lack of correlation which can be seen in table 3, between the presence of complement fixing antibodies and high titer or frequent neutralizing antibodies in the acute serums.

The relationship between the presence of neutralizing antibody measurable at 1:2 dilution in the acute serum specimen and the occurrence of a heterotypic rise is examined in table 7. This table shows that of the total of 477 determinations for heterotypic rises, 103 were observed. Forty-two heterotypic rises occurred in the presence of acute antibody at the level of 1:2 or greater while 61 occurred in the absence of acute antibody. Since acute antibody was present 178 times the percentage of heterotypic rises in its presence was 24 compared to 20%

TABLE 7.—*The relation of neutralizing antibody in the acute serum and the occurrence of fourfold heterotypic rises against adenovirus types 1 through 10 in paired serums from 53 persons with acute respiratory illness and adenovirus isolated.*

Adenovirus type isolated	Total number of tests	Acute neutralizing antibody present*			No acute neutralizing antibody		
		Rise	No rise	Total	Rise	No rise	Total
3	45	1	12	13	3	29	32
4	225	21	55	76	31	118	149
7	207	20	69	89	27	91	118
Total	477	42	136	178	61	238	299
Percent		24			20		

* 1:2 or greater.

rises in its absence. Table 7 also shows that there were no differences in the association of acute antibody with heterotypic rises by adenovirus type isolated.

DISCUSSION

The isolation of an adenovirus in association with a complement fixing antibody rise suggests that the 53 patients studied suffered an infection with the isolated virus. That such an infection had occurred in 51 of these persons was confirmed by significant, neutralizing antibody rises against the prototype virus isolated. The failure to find a neutralizing antibody rise against the type isolated in two persons remains unexplained although the presence of neutralizing antibody in their acute serum samples against the isolated type may be involved. Five of the 53 individuals had some neutralizing antibody against the isolated type with three showing more than a fourfold titer rise in the convalescent serums. On the basis of the data available it cannot be assumed that the low titer neutralizing antibody failed to protect against illness, since it is not certain that the antibody was present at the time of onset of infection. It is possible that these antibodies may have been produced by the current infection.

Although it must be emphasized that these 53 persons represent a special group infected with adenovirus, in general, the pattern of acute antibody to types 1 through 6 found in these young adult males was similar to that reported by Huebner and associates.[13] The relatively large number of acute serums with neutralizing antibody against adenovirus types 1, 2, 3, 5 and 6 may be considered a reason that only a few infections were caused by any of these types.[10] The relatively few acute serums with type 4 and 7 antibodies is consistent with the frequent infections with types 4 and 7 in these persons. Although the patients in this study would appear to be susceptible on the basis of absence of acute antibody to adenovirus types 8, 9 and 10, these agents were apparently not present in the populations studied as they have not been isolated.[10]

The more rapid fall after infection in adenovirus complement fixing antibody titers than neutralizing antibody titer reported by Cockburn and associates[14] may offer an explanation for the lack of correlation between the presence of neutralizing antibodies and complement fixing antibodies in the acute serum samples.

The finding of heterotypic neutralizing antibody responses as great or greater than the homotypic response occurred frequently enough to make it impractical to attempt to tell the infecting type on the basis of the neutralization test alone. In the data presented, these higher heterotypic responses were more frequently associated with type 7 infection. Hilleman et al[9] found some persons infected with type 7 adenovirus who also had a higher heterotypic neutralizing antibody response.

The cause of the relatively frequent heterotypic neutralizing responses observed in these studies is not apparent to us. Admixture of viral antigens is ruled out by the failure to find impor-

13. Huebner, R. J., Rowe, W. P., Ward, T. G., Parrott, R. H. and Bell, J. A. 1954, Adenopharyngeal-conjunctival agents. A newly recognized group of common viruses of the respiratory system. New Eng. J. Med. **251**: 1077–1086.

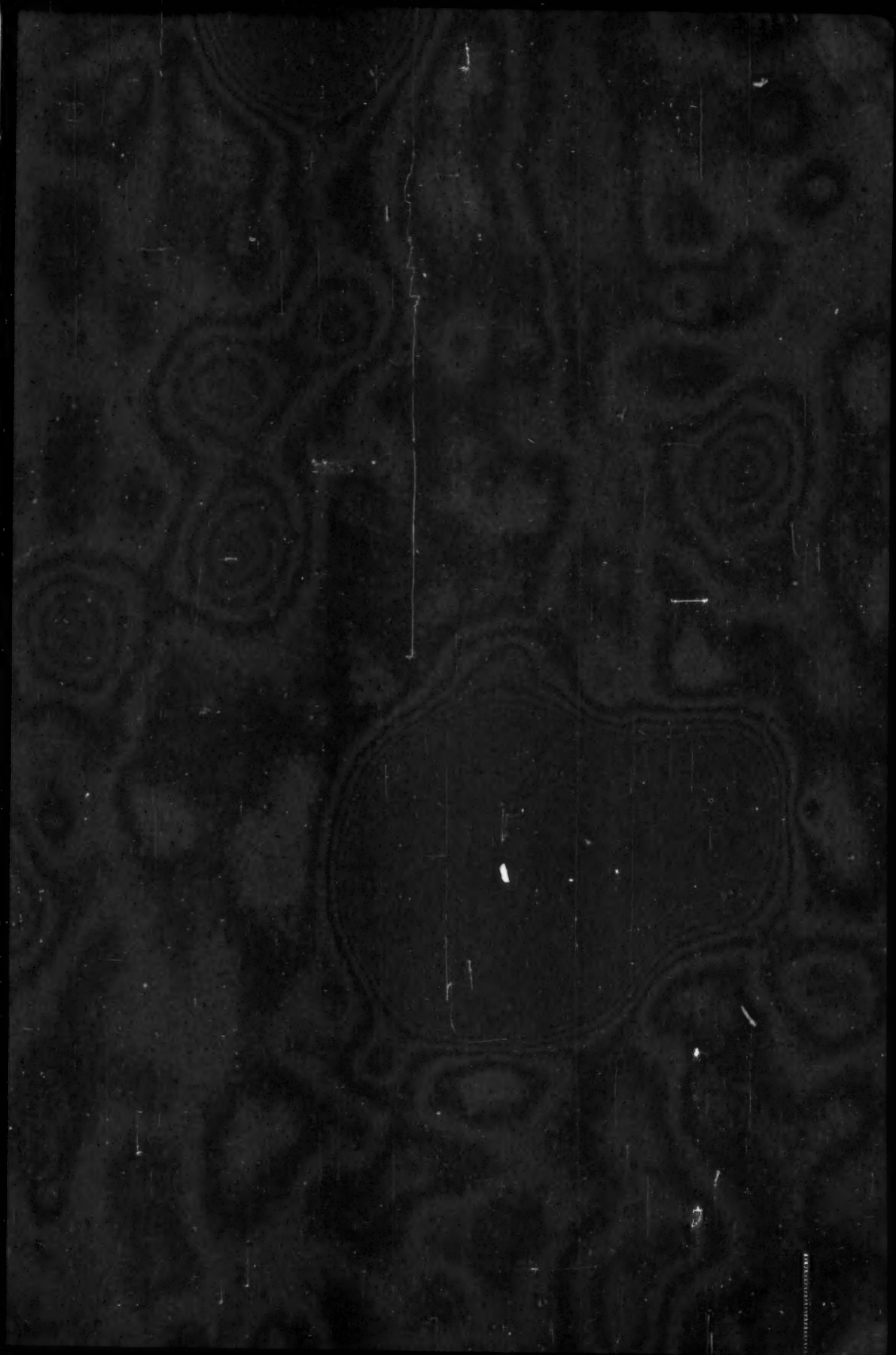
14. Cockburn, T. A., Rowe, W. P. and Huebner, R. J. 1956, Relationship of the 1951 Greeley, Colorado, outbreak of conjunctivitis and pharyngitis to Type 3 APC virus infection. Am. J. Hyg. **63**: 250–260.

tant cross reactions in the rabbit antiserums. No evidence has ever been found of infection with more than one adenovirus at the same time. Previous infection with other adenovirus types giving a predisposition to heterotypic rises would seem a likely explanation. However, no evidence that such a recall phenomenon played a role could be found in the data. Heterotypic rises were not seen significantly more frequently when neutralizing antibody was present at least at the 1:2 dilution in the acute serum than when no antibodies were found. This is further borne out by the fact that the two antigens associated with the greatest frequency of heterotypic responses had in one case (type 3) frequent acute antibodies and in the other (type 4) infrequent. In the case of type 4 it is unlikely that neutralizing antibody which was unmeasurable by the method employed was present since the population group involved was highly susceptible to infection with this type.

Although heterotypic responses with antigen types 2 and 6 were associated with infection with adenovirus type 4, while heterotypic neutralizing response to type 8 was most frequent in type 7 infection, no assurity that this represents a pattern of cross reaction can be taken from the numbers involved. Further experience with neutralizing antibody response in persons infected with adenovirus may clarify the nature of these cross reactions and provide an explanation for the frequent heterotypic rises.

SUMMARY

The neutralizing and complement fixing antibody response to adenovirus antigens is reported for 53 patients suffering acute respiratory illnesses and having an adenovirus, either type 3, 4 or 7, isolated from their upper respiratory tracts. A complement fixing antibody titer rise of fourfold or greater between the acute and convalescent serum samples was demonstrated for each of the patients. Fifty-one of the patients had fourfold rises in neutralizing antibody against the isolated virus prototype. The two patients failing to show a rise in neutralizing antibodies had present in their acute serum samples neutralizing antibody against the virus type isolated. In addition to neutralizing antibody rises against the homotypic virus antigen, tests with adenovirus types 1 through 10 showed heterotypic rises with each antigen type in from 13 to 44% of the serum pairs. Neutralizing antibodies were found in the acute serum samples frequently with antigen types 1, 2, 3, 5 and 6, infrequently with types 4, 7, 8, and 10, and not at all with type 9. No relationship between the presence of antibody in the acute serum and the occurrence of a heterotypic neutralization antibody rise could be demonstrated.



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